JYVÄSKYLÄ STUDIES IN BIOLOGICAL AND ENVIRONMENTAL SCIENCE
323

Marina Mustonen

Genetic Diversity of a Clonal Earthworm

Gene Expression Variation and Impacts on Decomposition in Metal Contaminated Soil

JYVÄSKYLÄN YLIOPISTO

JYVÄSKYLÄ STUDIES IN BIOLOGICAL AND ENVIRONMENTAL SCIENCE 323

Marina Mustonen

Genetic Diversity of a Clonal Earthworm

Gene Expression Variation and Impacts on Decomposition in Metal Contaminated Soil

Esitetään Jyväskylän yliopiston matemaattis-luonnontieteellisen tiedekunnan suostumuksella
julkisesti tarkastettavaksi yliopiston Vanhassa juhlasalissa (S212),
joulukuun 2. päivänä 2016 kello 12.

Academic dissertation to be publicly discussed, by permission of
the Faculty of Mathematics and Science of the University of Jyväskylä,
in building Seminarium, hall S212, on December 2, 2016 at 12 o'clock noon.

UNIVERSITY OF JYVÄSKYLÄ

JYVÄSKYLÄ 2016

Genetic Diversity of a Clonal Earthworm

Gene Expression Variation and Impacts on Decomposition in Metal Contaminated Soil

JYVÄSKYLÄ STUDIES IN BIOLOGICAL AND ENVIRONMENTAL SCIENCE 323

Marina Mustonen

Genetic Diversity of a Clonal Earthworm

Gene Expression Variation and Impacts on Decomposition in Metal Contaminated Soil

UNIVERSITY OF JYVÄSKYLÄ

JYVÄSKYLÄ 2016

Editors
Jari Haimi
Department of Biological and Environmental Science, University of Jyväskylä
Pekka Olsbo, Ville Korkiakangas
Publishing Unit, University Library of Jyväskylä

Jyväskylä Studies in Biological and Environmental Science
Editorial Board

Jari Haimi, Anssi Lensu, Timo Marjomäki, Varpu Marjomäki
Department of Biological and Environmental Science, University of Jyväskylä

URN:ISBN:978-951-39-6834-2
ISBN 978-951-39-6834-2 (PDF)

ISBN 978-951-39-6833-5 (nid.)
ISSN 1456-9701

Jyväskylä University Printing House, Jyväskylä 2016

ABSTRACT

Mustonen, Marina
Genetic diversity of a clonal earthworm: gene expression variation and impacts on decomposition in metal contaminated soil
Jyväskylä: University of Jyväskylä, 2016, 49 p.
(Jyväskylä Studies in Biological and Environmental Science
ISSN 1456-9701; 323)
ISBN 978-951-39-6833-5 (nid.)
ISBN 978-951-39-6834-2 (PDF)
Yhteenveto: Geenien ilmentymisen vaihtelu metsälierolla ja lajin geneettisen diversiteetin vaikutus ekosysteemin toimintaan metallisaastuneessa maassa
Diss.

Biodiversity affects ecosystem functioning positively. Metal-contamination diminishes species diversity and ecosystem function depends on the remaining species, and the genotypes within them. I studied how the clonally reproducing earthworm *Dendrobaena octaedra* responds to soil metal contamination and how its genetic diversity affects decomposition. Expression of the gene for the metal tolerance protein metallothionein was compared between populations with or without metal exposure history: with exposure history expression was high and stable and without there was a slow response. Adaptation to metal contamination likely explains the differences. Clonality of *D. octaedra* was verified with microsatellite markers. However, there was considerable gene expression variation both between and within genotypes, highlighting the need to consider such variation in comparative gene expression studies. The main study question focused on if genetic diversity is important for ecosystem functioning in metal-contaminated soil. I manipulated genetic diversity of a key decomposer, *D. octaedra* in a microcosm experiment. Indications of positive effects of higher genetic diversity on decomposition were found, especially in the contaminated conditions. However, the differences between the treatments often depended on which genotype was in the low diversity treatment, suggesting that traits of the genotypes might be more important than the level of genetic diversity for ecosystem functioning. An alternative technique to the real-time quantitative PCR, droplet digital PCR, was tested for gene expression measurements, and found to be a promising technique.

Keywords: Genetic diversity; decomposition processes; *Dendrobaena octaedra*; metal contamination; gene expression; clones.

Marina Mustonen, University of Jyväskylä, Department of Biological and Environmental Science, P.O. Box 35, FI-40014 University of Jyväskylä, Finland

Author's address

Marina Mustonen
Department of Biological and Environmental Science
P.O. Box 35
FI-40014 University of Jyväskylä
marina.s.mustonen@jyu.fi

Supervisors

Academy Researcher K. Emily Knott
Department of Biological and Environmental Science
P.O. Box 35
FI-40014 University of Jyväskylä

University Lecturer Jari Haimi
Department of Biological and Environmental Science
P.O. Box 35
FI-40014 University of Jyväskylä

Reviewers

University Lecturer Juha Mikola
Department of Environmental Sciences
University of Helsinki
Niemenkatu 73
15140 Lahti

Professor Stephen Sturzenbaum
Faculty of Life Sciences & Medicine
Analytical and Environmental Science Division
150 Stamford Street, London

Opponent

Professor Nico van Straalen
Vrije Universiteit Amsterdam
De Boelelaan 1085
1081 HV Amsterdam

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

1 INTRODUCTION 5
1.1 Biodiversity and ecosystem functioning 5
1.2 Diversity of soil invertebrates 6
1.3 Clonality and genetic diversity 7
1.4 Effect of metal contamination on diversity 8
1.5 Effects of metals on ecosystem functioning via changes in diversity . 9
1.6 Measuring effects of biodiversity on ecosystem functioning 9
1.7 Gene expression as a phenotypic response 10
1.8 Aims of the thesis 12

2 MATERIALS AND METHODS 15
2.1 Study species *Dendrobaena octaedra* 15
2.2 Earthworm collection and establishing cultures 16
2.3 Microsatellite markers and genes used for gene expression measurements 16
2.4 Soil used in the experiments 17
2.5 DNA and genotyping, RNA extractions, qPCR and ddPCR 18
2.6 Soil microcosm measurements (III) 19
2.7 Experimental setups, data handling and statistical analyses 19
2.7.1 Metal exposure experiment (I) 19
2.7.2 Variation in gene expression in clones (II) 19
2.7.3 Effect of genetic diversity on decomposition processes (III) .. 20
2.7.4 Using droplet digital PCR technique for gene expression measurements (IV) 21
2.7.5 Raw data handling and general statistics (I, II, III, IV) 21

3 RESULTS AND DISCUSSION 22
3.1 Metallothionein gene expression differs in earthworm populations with different exposure history (I) 22
3.2 Variation in gene expression within clones of the earthworm *Dendrobaena octaedra* (II) 24
3.3 Genetic diversity of *D. octaedra* affects ecosystem functioning (III) .. 27
3.4 Using droplet digital PCR for gene expression measurements (IV) . 30

4 CONCLUSIONS 32

Acknowledgements 34

YHTEENVETO (RÉSUMÉ IN FINNISH) 36

REFERENCES 39

LIST OF ORIGINAL PUBLICATIONS

This thesis in based on the following original papers, which will be referred to in the text by their Roman numerals.

I Mustonen, M., Haimi, J., Väisänen, A. & Knott, K.E. 2014. Metallothionein gene expression differs in earthworm populations with different exposure history. Ecotoxicology 23: 994-1007.

II Mustonen, M., Haimi, J., Kesäniemi, J, Högmander, H. & Knott, K.E. 2016. Variation in gene expression within clones of the earthworm *Dendrobaena octaedra*. Submitted manuscript.

III Mustonen, M., Haimi, J. & Knott, K.E. 2016. Genetic diversity of an earthworm affects ecosystem functioning. Submitted manuscript.

IV Mustonen, M. & Knott, K.E. 2016. Going digital - a new technique for quantifying gene expression in molecular ecology. Manuscript.

The table shows the contributions to the original papers.

	I	II	III	IV
Original idea	KEK, JH	KEK, JH, MM	KEK, JH	KEK, MM
Data	MM, KEK, JH, AV	MM, KEK, JH, JK	MM, KEK, JH	MM, KEK
Analyses	MM, KEK, JH	MM, KEK, JH, HH	MM, KEK, JH	MM, KEK
Writing	MM, KEK,JH	MM, KEK, JH, JK, HH	MM, KEK,JH	MM, KEK

MM = Marina Mustonen, KEK = K. Emily Knott, JH = Jari Haimi, AV = Ari Väisänen, JK = Jenni Kesäniemi, HH = Harri Högmander

1 INTRODUCTION

1.1 Biodiversity and ecosystem functioning

Ecosystem functioning encompasses biogeochemical processes that affect the amounts of materials (e.g. organic matter) and the rates of cycling materials and flow of energy within a given ecosystem, for example primary production and decomposition (Hooper *et al.* 2005). Biodiversity includes different components, e.g. richness, relative abundance and composition, and can be measured on different levels e.g. genetic variation, species diversity, functional variation and biome distribution (Hooper *et al.* 2005). Ecosystem functioning and biodiversity are positively correlated: with more diversity there can be more complementation in ecological niches, which enhances resource use, and high-functioning species are more likely to be found from a more diverse pool of species (Gravel *et al.* 2011, Hooper *et al.* 2012, Harrison *et al.* 2014, Duncan *et al.* 2015, Evans 2016). Ecological processes affect evolutionary change, but it has been demonstrated that evolutionary processes, e.g. diversification of organisms, can also affect ecosystem functioning, and lead to two-way feedbacks on the diversity-functioning relationship (Harmon *et al.* 2009, Bassar *et al.* 2010, Matthews *et al.* 2011, Barraclough 2015).

Genetic diversity, an important level of biodiversity, can have significant effects on ecological processes, even rivaling the effects of species diversity (Hughes *et al.* 2008, Matthews *et al.* 2011). There are indications that genetic diversity is especially important in dominant or functionally important species, and in highly variable environments or when the environment is undergoing changes caused by human activity (Hughes *et al.* 2008, Matthews *et al.* 2011). The relationship between genetic diversity and ecosystem functioning is mostly studied in plants, with the ecosystem parameter often being primary production (plant biomass), but also the effect of genetic diversity on e.g. intraspecific competition, stability and recovery from disturbance have been studied (Reusch and Hughes 2006, Hughes *et al.* 2008, Avolion *et al.* 2014, Hughes 2014, Schöb *et al.* 2014, Prieto *et al.* 2015). Positive effects of genetic diversity on ecosystem

functioning are often found (e.g. Wymore *et al.* 2011), but it can depend on what kind of genetic diversity is studied. In a meta-analysis, Whitlock (2104) found that there was a positive correlation between intraspecific diversity and ecological functioning in plants when looking at adaptive genetic diversity (e.g. genotypic richness), but for neutral genetic diversity (variation in selectively neutral loci) the positive correlation was noticed only in specific conditions. Effects of genetic diversity on ecosystem processes is less studied in animals, but there are few studies showing that higher genetic diversity has a positive effect on some ecological parameters e.g. on survival, growth and fecundity in a marine invertebrate *Bugula neritina* (Aguirre and Marshall 2012), and on crowding in black surfperch *Embiotica jacksoni* (Johnson *et al.*2016).

1.2 Diversity of soil invertebrates

Soil invertebrates participate in many ecosystem functional processes, e.g. building and maintaining porosity of the soil, controlling microbial activity, protecting plants from some pests and accelerating plant growth (Lavelle *et al.* 2006). Plants provide organic material to the soil food webs through roots and dead litter that the soil invertebrates can use directly as energy sources (e.g. decomposing dead organic matter) and indirectly, by consuming other soil organisms utilizing the organic material (Lavelle *et al.* 2006, Sylvain and Wall 2011). Soil invertebrates participate in recycling nutrients back to the plants through processes that transform organic compounds to inorganic nutrients, i.e. nutrient mineralization (Lavelle *et al.* 2006, Sylvain and Wall 2011). Soil invertebrates affect nutrient cycling mostly through comminution of litter and increasing microbial activity through selective feeding, but also by affecting the vertical dispersion of organic matter (transporting litter from the top layer to deeper layers of the soil) and creating structures that act as incubators of microbial activity (Lavelle *et al.* 2006). Among soil invertebrates, earthworms are often key-species and contribute considerably to soil structure and nutrient cycling (Edwards and Bohlen 1996, Spurgeon *et al.* 2003, Lavelle *et al.* 2006, Sturzenbaum *et al.* 2009).

Diversity of organisms in soil is often very high, and there are indications that biodiversity has a significant impact on decomposition and nutrient cycling, but the importance of genetic, species and functional diversity of soil organisms on ecosystem functioning is not fully understood (Hättenschwiler *et al.* 2005, Gessner *et al.* 2010, Bardgett and van der Putten 2014). A common view is that there is functional redundancy in soil communities, and it is the species' traits (the function of the species) that are more important than species diversity *per se*, meaning that community composition, e.g. presence of key-species and functionally different groups is what affects the soil decomposition processes (Laakso and Setälä 1999, Ekschmitt *et al.* 2001, Cragg and Bartgett 2001, Heemsbergen *et al.* 2004, Eisenhauer and Schädler 2011, Bardgett and van der Putten 2014). On the other hand, other studies have found that also species diversity is important for ecosystem functioning (Scheu *et al.* 2002, Zimmer *et al.* 2005,

Adejuyigbe *et al.* 2006, Eisenhauer *et al.* 2012, Wagg *et al.* 2014, Jung *et al.* 2016). In earthworms, species diversity is often not very high within a habitat, but it has been shown that both functional diversity and species diversity of earthworms affect ecosystem functioning (Scheu *et al.* 2002, Hättenschwiler *et al.* 2005, Sturzenbaum *et al.* 2009).

1.3 Clonality and genetic diversity

Asexual reproduction is not very common in animals: on one hand producing offspring via asexual reproduction is faster and more efficient, since the individual does not have to spend time finding a mate and more energy can be allocated to production of offspring, but on the other hand, with asexual reproduction, genetic diversity could be diminished and with it the potential to adapt to changed environments (Butlin 2002, Neiman and Schwander 2011, Annila and Annila 2012). Asexual lineages are thought often to be short lived, an evolutionary dead-end in the long run, with slower capabilities for adaptation and greater accumulation of deleterious mutations, whereas recombination and segregation of chromosomes in sexual lineages maintain higher genetic diversity, on which natural selection can act (Butlin 2002, Neiman and Schwander 2011, Annila and Annila 2012). In earthworms, many species are hermaphrodites and reproduce through cross-fertilization (or in some cases through self-fertilization), but parthenogenetic reproduction, in which the egg is not fertilized, is also common (Diaz Cosin *et al.* 2010). Parthenogenetic reproduction can happen with meiosis (automixis), in which recombination causes offspring to have some genetic differences from their parent (often still called clones), or mitosis (apomixis), in which offspring are true clones of their parent (Diaz Cosin *et al.* 2010). The definition of a clone is under some debate, ranging from simply referring to all individuals in asexual lineages as clones to requiring that clones must have genetic fidelity among individuals. Often there is some genetic variation between individuals even in apomictic lineages, thus the requirement that clones are genetically identical is rarely met (Lushai and Loxdale 2002). Clone is still a commonly used concept to describe individuals in asexual lines, and I am also using it to describe the individuals originating from a single founder.

Clonal diversity, i.e. genetic diversity in parthenogenetic animals, has been found to vary: some species or lineages have low clonal diversity, whereas others have high diversity (Butlin *et al.* 1998, Terhivuo and Saura 2006). Sources of clonal diversity have been suggested to be accumulation of mutations, multiple origins of asexual lineages, occasional hybridization with sexual individuals, somatic recombination and automixis (Butlin et. al. 1998). Environmental change and ecological differentiation could maintain the variation to some extent (Butlin et. al. 1998), and high dispersal can contribute to local diversity (Terhivuo and Saura 2006). In earthworms, genetic diversity is often high (Sturzenbaum *et al.* 2009). My study species, *Dendrobaena octaedra*, reproduces

through mitotic parthenogenesis, thus the offspring are assumed to be clones of their parent (Omodeo 1955, Hongel and Terhivuo 1989, Terhivuo and Saura 1990), but high genetic variation has still been found in *D. octaedra* populations (Hansen *et al.* 2006, Haimi *et al.* 2007, Simonsen *et al.* 2008, Knott and Haimi 2010).

Clones or inbred lines are quite often used in gene expression comparisons, e.g. in ecotoxicological studies in an attempt to diminish variation that could confuse interpretation of the results. However, a potential problem could be that individuals assumed to be clones could actually have unexpected genetic differences, e.g. due to mutations, that affect gene expression, which can lead to different interpretations depending of which genotype is used (Dalziel *et al.* 2009, Nota *et al.* 2013).

1.4 Effect of metal contamination on diversity

Metal contamination, caused by human activities such as agriculture, metallurgy (e.g. mining and smelting), energy production, transportation, and waste disposal (Bradl 2005), can have negative impact on organisms through the production of reactive radicals that induce genotoxic damage, lipid peroxidation and depletion of proteinsulfhydryls (Valko *et al.* 2005). Even though some metals are essential for organisms (e.g. Cu and Zn are needed for metabolic and signaling pathways), these can become toxic in high concentrations. On the other hand, some non-essential metals (e.g. Cd and Hg) can be toxic even at low concentrations (Valko *et al.* 2005, Roelofs *et al.* 2008). Since metals are easily bound especially to the organic matter, accumulating in the litter layer, and are a persistent stress in soils, earthworms are likely be affected by metals. Thus, earthworms are often used as indicator organisms to monitor the effect of metals on ecosystem structure and functioning (Bengtsson *et al.* 1983, Lavelle *et al.* 2006, Nahmani *et al.* 2007, Roelofs *et al.* 2008, Simonsen *et al.* 2008). However, the effect of metal contamination on diversity might be difficult to detect. Noticeable loss or persistence of species might only be evident after a high-level and/or long-term contamination of the environment has occurred (Spurgeon *et al.* 2005a). Thus, molecular genetic approaches (e.g. expression of stress-related genes) have been used in ecotoxicological research to study the more immediate biological effects of metal contamination (Roelofs *et al.* 2008, Sturzenbaum *et al.* 2009).

Even though species diversity is often diminished in metal-contaminated environments, genetic diversity in the species that remain can be very little affected by metals. There are some studies that show negative impact of metals on genetic diversity (Ross *et al.* 2002, van Straalen and Timmermans 2007, Ungherese *et al.* 2010), but also many cases where metal contamination affected the genetic diversity only a little or not at all (Haimi *et al.* 2006, Costa *et al.* 2012, Costa *et al.* 2013), including my study species, *Dendrobaena octaedra* (Haimi *et al.* 2007, Simonsen *et al.* 2008, Knott and Haimi 2010). Even higher genetic diversity

in metal contaminated sites than reference sites can be found, as with the beetle *Staphylinus erythropterus* (Giska *et al.* 2015).

1.5 Effects of metals on ecosystem functioning via changes in diversity

Since not all organisms can cope with metal toxicity, metal contamination can decrease species diversity (Spurgeon and Hopkin 1999, Johnston and Roberts 2009, Kozlov and Zvereva 2011), which can have major, and often detrimental, effects on ecosystem functioning (Hooper *et al.* 2012, Johnston *et al.* 2015). Metals can affect ecosystem functioning directly by decreasing activity of microbes and diversity of decomposer animals, but also indirectly by decreasing primary production and changing plant community structure, which has a negative effect on microbial and faunal communities (Niemeyer *et al.* 2012). Fernandes and colleagues (2011) found that functional traits within a fungal species (cadmium sensitive or cadmium tolerant functional types) affected the species diversity-ecosystem functioning relationship: species diversity had a positive effect on fungal biomass and dead plant material decomposition in uncontaminated conditions but in contaminated conditions the positive effect of species diversity was decreased when the Cd-sensitive functional type (no metal contamination history) was used but remained when the Cd-tolerant functional type (metal contamination history) was used. In metal contaminated environments, biodiversity is expected to be especially important for ecosystem functioning (Farina *et al.* 2003). Similarly, biodiversity is particularly important for ecosystem functioning in species-poor communities (Ekschmitt *et al.* 2001).

Genetic diversity might not be as affected by metals as is species diversity, but more studies are needed to understand how metals affect ecosystem functioning through genetic diversity. Nota and colleagues (2013) found that different genotypes of *Folsomia candida*, originally from the same line and previously assumed to have the same genotype, had a different responses to cadmium, with one being more sensitive than the other lineage. Since species diversity is diminished in metal contaminated conditions, it could be hypothesized that the genetic diversity of the species that persist becomes more important for the ecosystem functioning, similarly to species diversity being more important in species-poor, rather than species-rich communities (Ekschmitt *et al.* 2001).

1.6 Measuring effects of biodiversity on ecosystem functioning

Ecosystem functioning can be measured indirectly using structural parameters (e.g. species richness and composition, biomass and faunal density) or by directly measuring functional parameters, such as organic material breakdown,

respiration and nitrification (van Straalen 2002, Niemeyer *et al.* 2012). The processes involved in ecosystem functioning are often studied by measuring proxies for them, e.g. biomass of plants to measure aboveground primary production and root biomass to determine belowground primary production, decomposition of litter bags to measure organic material breakdown, CO_2 production to measure biological activity or concentration of ammonium nitrate to measure nitrogen mineralization (van Straalen 2002, Niemeyer *et al.* 2012, Meyer *et al.* 2015). It has been proposed that many parameters, both structural and functional, should be measured to get an overall understanding how an ecosystem is functioning (van Straalen 2002, Niemeyer *et al.* 2012, Meyer *et al.* 2015).

Ecosystem functioning can be studied in the field, where many parameters can be measured, e.g. species diversity, density and composition, vegetation cover and biomass of plants, animals and microbes, or functioning can be studied in semi-controlled experiments, which can be conducted to find out the response of organisms e.g. to a contaminant. Ecosystem functioning can also be studied with microcosms, in which conditions, e.g. level of contamination or temperature, can be controlled. Microcosms have the benefit that experiments are easier to replicate, and that the measurement of some parameters, e.g. CO_2 production, nutrient mineralization and growth and mortality of organisms, is simpler and more reliable (van Straalen 2002, Meyer *et al.* 2015). In field studies structural parameters can be more comprehensively studied, but processes are difficult to study in detail in the field because of all the interactions and uncontrollable conditions. Functional parameters, e.g. rates of processes, such as nutrient mineralization, are easier to study in microcosms, but experimental time is limited and some important interactions might be missed because of the simpler experimental design (Verhoef 1996, van Straalen 2002, Meyer *et al.* 2015).

1.7 Gene expression as a phenotypic response

Measuring gene expression can be very useful when studying the response of an organism to altered conditions, e.g. metal contamination. Effect of metal contamination can be measured from the expression of proteins that function in homeostasis and the detoxification of metals, e.g. metallothioneins (Cortet *et al.* 1999, Weeks *et al.* 2004). Metallothioneins are small (6-8 kDa), hydrophilic metal-binding proteins, that are found in many organisms (Dallinger 1996, Janssens *et al.* 2009), and are important for metal management and tolerance through detoxification of non-essential metals and regulation of essential ones (Cortet *et al.* 1999, Valko *et al.* 2005). Clear time- and dose–response relationships between metal exposure and metallothionein expression or concentration have been found in many studies, thus metallothioneins are considered to be good biomarkers for the effects of metal contamination (Dallinger *et al.* 1996, Cortet *et al.* 1999, Gruber et. al. 2000, Brulle *et al.* 2006, Demuynck *et al.* 2007). Sturzenbaum (1998) first identified and described metallothioneins in earthworms and now their concentration and gene expression in response to metals have been stud-

ied in many earthworm species (Marino *et al.* 1998, Lukkari *et al.* 2004a, Galay Burgos *et al.* 2005, Spurgeon *et al.* 2005a, 2005b, Brulle *et al.* 2006, 2007, 2011, Demuynck 2006, 2007, Bundy *et al.* 2008, Gruber *et al.* 2000, Asensio *et al.* 2013, Calisi *et al.* 2013), including my study species, *Dendrobaena octaedra* (Fisker *et al.* 2013).

In addition to studying the effects of metal contamination, and the effect of other stressors, e.g. cold, heat, drought and salinity (Roelofs *et al.* 2008), gene expression measurements can also be used to study other questions, such as the role of gene expression in speciation and adaptation, or the effect of natural selection on gene expression variation (Emerson and Li 2010, Romero *et al.* 2012). For example, differences in gene expression can be measured between populations with different histories, or response of different groups of individuals, e.g. differences in metabolism between lineages cultured in different conditions.

There is high variation in gene expression and it is regulated at many levels, helping organisms acclimate and adapt to changing environments (Levine and Tjian 2003, Lopez-Maury *et al.* 2008, Viney and Reece 2013). Gene expression differs between different tissues and at different stages of the organisms' life (Rosa *et al.* 2014, Rue and Arias 2015). Because variation can complicate interpretations of population responses to a specific condition or contaminant, some experimental designs reduce variation by examining only a single clonal genotype or inbred lines, and others use pools of different individuals when determining gene expression levels (Dudycha *et al.* 2012). Although rarely reported, there is also variation in gene expression among genetically identical individuals, possibly due to physiological or epigenetic differences (Wolff 1996, Wong *et al.* 2005, Pritchard *et al.* 2006, Rosa *et al.* 2014, Ong-abdullah *et al.* 2015, Rue and Arias 2015). The variation in gene expression has to be taken into consideration when gene expression is examined as a phenotypic response. For example, when using one clonal genotype, conclusions about the response can only be drawn for that specific genotype, or when using pools of individuals, important variation might not be detected (Dudycha *et al.* 2012).

Gene expression measurements have many technical challenges that need to be resolved before the results of the measurements can be considered reliable (Bustin *et al.* 2009). For example, for all gene expression assays, specificity of the primers, efficiency of the primers, variation in the RNA extraction or amount of inhibitors, variation in pipetting precision, and variation between different runs should be checked and reported (Bustin *et al.* 2009). Moreover, there is a need to normalize the target expression to control the non-biological variation (Bustin *et al.* 2009). Gene expression can be measured e.g. with the real-time quantitative polymerase chain reaction (qPCR) technique, in which cDNA reverse transcribed from mRNA is used, and the quantity of the target sequence is determined by measuring the amplification of that sequence in each PCR cycle with the help of fluorescent dyes (Bustin 2000). Another technique that can be used for measuring gene expression is droplet digital polymerase chain reaction (ddPCR), which partitions the sample into 10 000-20 000 droplets where PCR is done: the quantity of the target sequence is determined from the amount of

droplets in which amplification has happened (positive droplets) relative to the amount of droplets in which amplification has not happened (negative droplets) (Hindson *et al.* 2011). MIQE guidelines have been published for both qPCR and dPCR (Minimum Information for Publication of Quantitative Real-Time/Digital PCR Experiments) and following them is recommended for reliable and comparable data from different laboratories and study systems (Bustin *et al.* 2009, Huggett *et al.* 2013). ddPCR is more sensitive to smaller changes in expression and less affected by technical variation (Huggett *et al.* 2013), but it has not yet been used much in molecular ecology studies, thus there is a need for testing this technique with samples with ecological relevance.

1.8 Aims of the thesis

The main question of this thesis was how genetic diversity affects ecosystem functioning. To provide more information on the diversity-functioning relationship, I studied the clonally reproducing earthworm *Dendrobaena octaedra*. This species was chosen for several reasons. Because *D. octaeda* reproduces clonally, it was possible to study both low genetic diversity (groups of clones) as well as high genetic diversity (mixtures of different genotypes) populations. Because *D. octaedra* is an important decomposer species, I had the chance to study the effect of its genetic diversity on a key ecosystem functioning process, decomposition. Because *D. octaedra* can persist in even highly metal contaminated environments, and since the effect of genetic diversity on ecosystem functioning could be different in metal contaminated environments than in uncontaminated environments, I could study the effect of metal contamination on the genetic diversity-ecosystem functioning relationship in my thesis.

I first gathered more information about the response of *D. octaedra* to metal contamination and effect of population history on that response (I). Expression of the gene involved in metal tolerance, metallothionein, was measured from individuals sampled from populations with different metal exposure history, and an experiment with different exposure times and metal concentrations was conducted. My study questions were: 1. how does population history affect the metallothionein gene expression response of *D. octaedra,* and 2. is there a time- and dose-response in the expression of metallothionein? I hypothesized that earthworms originating from populations with different metal exposure histories would differ in expression of metallothionein (I).

Secondly, since genetically identical individuals were required for my experiments to study the effect of genetic diversity on ecosystem functioning, it was essential for me to verify that *D. octaedra* offspring are indeed clones of their parent (II). There has been some doubt whether *D. octaedra* reproduces solely through apomictic parthenogenesis, as originally described, given a previous study using allozyme marker variation by Simonsen and Holmstrup (2008). Also, since gene expression can be noisy (Viney and Reece 2013), it was important to study how much variation there can be between clones and if this

can influence the use of gene expression measurements as a measure of phenotypic response. Microsatellites were used to genotype *D. octaedra* offspring and their parents, and expression of several genes was measured to examine variation in gene expression both within and among genotypes in two generations of earthworms. The study questions were: 1. are *D. octaedra* offspring clones of their parent, and 2. how much variation in gene expression is within genotypes compared to between genotypes? I hypothesized that the microsatellites would confirm that offspring are clones of their parents, thus supporting mitotic parthenogenesis in *D. octaedra*, and that there would be less variation in gene expression within genotypes than between genotypes (II).

Thirdly, I studied the effects of genetic diversity on decomposition processes, by establishing a year-long microcosm experiment simulating two growing seasons in which the microcosms contained either low genetic diversity (individuals being clones) or high genetic diversity (individuals having different genotypes) of *D. octaedra* (III). The effect of genetic diversity on ecosystem functioning is not widely studied, and to my knowledge, the effect of genetic diversity of earthworms on decomposition processes has not been studied before. The effect of metal contamination on the relationship between genetic diversity and decomposition processes was studied by using Cu contaminated soil in half of the microcosms. I also included two diversity levels of other decomposer animals in the microcosm experiment: in addition to treatments manipulating genetic diversity of *D. octaedra,* some microcosms had only very few other species present and others had a diverse community of decomposer animals. CO_2 production, soil NH_4-N concentration, and growth, mortality, cocoon production and the expression of six genes of the earthworms were measured as functional and structural proxies for decomposition activity, metabolism and condition of the earthworms. Study questions were: 1. how does genetic diversity of a key decomposer affect the decomposition processes, 2. is the effect of higher genetic diversity on decomposition different in stressed (i.e. metal contaminated) than unstressed (i.e. uncontaminated) conditions, and 3. how does species diversity affect the relationship between genetic diversity of one focal species and ecosystem functioning? I hypothesized that decomposition activity and nutrient mineralization would be higher in the high genetic diversity treatment than in the low genetic diversity treatment, and that the difference between the diversity treatments would be larger in metal contaminated conditions. Since there are more functional groups contributing to the decomposition processes when species diversity is high, I expected that the difference between low and high genetic diversity treatments could be masked when there is high diversity of the other decomposer animals (III).

Lastly, since gene expression measurements are a significant part of all of my other studies (I, II, III), testing how a different technique from the traditional qPCR would work with my *D. octaedra* samples was important (IV). qPCR is a simple, fast and accurate technique for measuring gene expression, and I already had working assays for qPCR to study gene expression in *D. octaedra*, but ddPCR has some advantages that could make it more precise and sensitive for

measuring gene expression. The study questions were: 1. how repeatable and reproducible are gene expression measurements when using ddPCR, 2. how consistent are the results when comparing gene expression variation between two populations when using both qPCR and ddPCR techniques, and 3. what is the influence of different RNA extraction methods on the results of gene expression measurements with ddPCR? I hypothesized that there would be high repeatability and reproducibility, and that the two techniques would yield consistent results (IV).

I expected that studying the clonally reproducing key decomposer earthworm, *D. octaedra* would provide new information on the effect of contamination history on the response to metal contamination, the variation in gene expression and the effect of genetic diversity on decomposition, with potential implications for future ecotoxicological studies and studies about the link between genetic diversity and ecosystem functioning.

2 MATERIALS AND METHODS

2.1 Study species *Dendrobaena octaedra*

Dendrobaena octaedra is a small, epigeic earthworm, living and reproducing in the soil litter layer (e.g. Rundgren 1975, Terhivuo 1988). *D. octaedra* is an ecologically important decomposer animal, efficient in dispersal and adapting to new areas, and it can be the dominant earthworm species in northern coniferous forests and tundra (Edwars and Bohlen 1996, Terhivuo and Saura 2006). *D. octaedra* is often exposed to metals accumulated in boreal forest ecosystems, but it can persist in contaminated soils even with high concentrations of metals (Bengtsson *et al.* 1983, 1992, Rozen *et al.* 2003, 2006, Lukkari *et al.* 2004b, Simonsen *et al.* 2008, Tosza *et al.* 2010, Holmstrup *et al.* 2011). One way that *D. octaedra* copes with the metal contamination is by avoiding areas with the highest concentrations, which is possible because metals are heterogeneously distributed in soils (Lukkari and Haimi 2005). *D. octaedra* can also adapt to metal exposure (Fisker *et al.* 2011a, 2011b, 2013).

D. octaedra is hexaploid with six times 18 chromosomes (108 chromosomes), although cytologic studies have indicated that there can be variation in chromosome number (Omodeo 1955, Hongell and Terhivuo 1989). *D. octaedra* has a long generation time (mean maturation time is 21.2 months, Rozen 2006) and is expected to reproduce through apomictic (mitotic) parthenogenesis, producing offspring that are clones of their parent. Some question whether other mechanisms besides apomictic parthenogenesis could be used by *D. octaedra* were raised when Simonsen and Holmstrup (2008) found some differences among the offspring and their parent in allozyme markers, but genotyping *D. octaedra* with other genetic markers has not been attempted before. Despite their parthenogenetic reproduction, *D. octaedra* has high genetic diversity (Hansen *et al.* 2006, Haimi *et al.* 2007, Simonsen *et al.* 2008, Knott and Haimi 2010).

2.2 Earthworm collection and establishing cultures

D. octaedra were collected from one metal contaminated site and one uncontaminated site. The contaminated site in Harjavalta (South-West Finland, 61°18'50''N, 22°08'30''E) is birch dominated forest, and the main source of the metal contamination is a Cu-Ni smelter ca. 1 km from the sampling site. The Cu smelter has been operational since 1945 and the Ni smelter since 1960. Even though emissions were significantly reduced after new filtration was installed in the early 1990s, decades of emissions have led to the accumulation of metals in the soil (Salemaa 2003). The site in Jyväskylä (Central Finland, 62°12'39''N, 25°44'13''E) is spruce dominated forest on moraine soil and there is no history of metal emissions in Jyväskylä. Earthworms were collected by hand and transferred in buckets with soil from the sampling site to the laboratory, where they were kept at +15°C in climate chambers.

In the laboratory, some of the earthworms were used for RNA extraction right away and the rest were used in a metal-exposure experiment (I), or to establish different kinds of cultures. To study variation within clones (II) juvenile or sub-adult earthworms were put either singly or in pairs (to allow a possibility for mating) in separate glass jars (Ø 8 cm, with perforated lids). Part of the single-worm cultures and all pair-worm cultures were used for microsatellite genotyping to determine if the offspring produced in culture were clones and the remaining single worm cultures were used to provide earthworms to study gene expression in clones. For the study about the effect of genetic diversity on decomposition processes (III) cultures were established from a single juvenile or sub-adult earthworm, in glass jars (Ø 8 cm, with perforated lids), and when needed, the cultures were transferred to larger plastic jars (Ø 13 cm, with perforated lids) to avoid overcrowding. All cultures contained uncontaminated organic-rich soil and horse manure (for nutrition) and were maintained at 15 °C. The soil was changed every few months.

2.3 Microsatellite markers and genes used for gene expression measurements

Microsatellite markers were used to genotype the earthworms to make sure that offspring are clones of their parent (II) and to check the genotypes of the earthworms used in the experiments (II and III). The microsatellite loci were isolated following the FIASCO technique (Zane 2002) with some modifications (Grapputo 2006). Primers were designed for six microsatellite loci (DO1, DO2, DO3, DO4, DO5 and DO6), but one (DO5) did not amplify consistently and was not considered further.

For the gene expression measurements (I, II, III, IV), all primers, except those for the metal-tolerance gene metallothionein (MT) and reference genes

18S and 28S (described in I), and the gene for heat-shock protein HSP70 (Fisker *et al.* 2013), were designed based on a draft transcriptome of *D. octaedra* (shared by M. Holmstrup). These included genes expected to be involved in metabolism: aldo/keto reductase (AkRed), carbonyl reductase (CarRed), chitinase domain (ChitDo), chymotrypsin inhibitor (ChymInh), dehydrogenase (DeHyd), (similar to) fucosidase (Fuco), leucine aminopeptidase-like protein (Leuc), pyruvate dehydrogenase (Pyr), L-xylulose reductase (Xyl), dual specifity phosphatase 14 (DualPhos), glucose-regulated protein 94 (GlucReg), pancreatic amylase (PanAmy), and one gene for heat-shock protein 40 (HSP40), in addition to two potential reference genes, peptidylprolyl isomerase (PepIso) and tubulin (Tub). Expression of MT was measured in the study on metal-exposure (I), in the study on variation in expression between clones (II) and in the study on effects of genetic diversity on decomposition processes (III). Expression of CarRed, ChitDo, DeHyd, Fuco, Leuc, Pyr and Xyl were measured in the study about the droplet digital PCR technique for gene expression measurements (IV) and, together with expression of genes HSP40 and HSP70, in the study about variation in expression between clones (II). Expression of genes DualPhos, GlucReg and PanAmy were examined in the study about effects of genetic diversity on decomposition processes (III), and expression of genes AkRed and ChymInh were examined in three studies (II, III, IV). In most cases, the potential reference genes, (18S, 28S, PepIso and Tub) had too much variation and could not be used as references. 18S and 28S worked in I and IV with qPCR but not in II and III, and PepIso and Tub were not good reference genes.

2.4 Soil used in the experiments

Soil for the experiments was collected from an uncontaminated mature spruce forest in Jyväskylä, and it was used either without (I, II) or with some fallen birch leaves that were shredded and mixed well with the soil for additional food for earthworms (III). For certain experiments, the experimental soil was contaminated by adding copper chloride ($CuCl_2 \cdot H_2O$) or zinc chloride ($ZnCl_2$) to pure water, which was then mixed well with the soil. Pure water was added to the control soil. Three different concentrations of Cu or Zn (50, 100, 300 mg Cu kg^{-1} or 75, 150, 300 mg Zn kg^{-1}) were used in the metal exposure experiment (I) and a moderate concentration of Cu (100 mg kg^{-1}) was used in the study about gene expression in clones (II) and effect of genetic diversity on ecosystem functioning (III). The spiked soil was incubated for 7 days at 15 °C before start of the experiments.

2.5 DNA and genotyping, RNA extractions, qPCR and ddPCR

For genotyping (II, III), genomic DNA was extracted from piece of the earthworm using Qiagen chemistry (DNeasy kit reagents) and a Kingfisher magnetic processor (Thermo Fisher Scientific). Amplification of the microsatellite loci was performed separately for each locus in 10 µl reactions containing 1 µl of template DNA. See details of reaction mix and thermocycling protocol in II. Amplification products were separated using the ABI PRISM 3130xl with GeneScan 500 LIZ size standard and genotyped using GeneMapper 5 software (all Applied Biosystems).

Before RNA extraction (I, II, III, IV), the earthworms were allowed to empty their guts by keeping them two days in jars with moist paper towel, which was changed after one day to prevent coprophagy. The anterior end (ca. 5 mm) of each earthworm was cut using scissors or a scalpel, placed in a 1.5 ml microcentrifuge tube and immediately frozen in liquid nitrogen, after which it was stored in -80 °C until RNA extraction. The rest of the body was reserved for metal content analysis (I), which were measured with an ultrasound-assisted extraction method (Perkin Elmer Optima 8300 using inductively coupled plasma optical emission spectrometry), or stored in -80 °C. Aurum Total RNA mini kit (Bio-Rad) was used for RNA extraction (I, II, III, IV) and the manufacturer's protocol was followed, including a DNase treatment. RNA extraction using Trizol (Thermo Fisher Scientific) together with the Direct-zol kit (Zymo) was also tested (IV). RNA concentration was determined with the Qubit RNA Assay Kit and Qubit Fluorometer (Invitrogen, Thermo Fisher Scientific). In most studies (I, II, III, IV) a concentration of 35 µg/mL of RNA was used for cDNA synthesis, for which the iScript cDNA synthesis kit (Bio-Rad) was used following the manufacturer's protocol**.** cDNA was then diluted 1:5. When comparing the Aurum and Trizol RNA extraction methods (IV) 75ng of RNA was used for cDNA synthesis. cDNA was then diluted 1:5.

Real-time quantitative polymerase chain reactions (qPCR; I, II, III, IV) were done with IQ SYBR green supermix (Bio-Rad). qPCR conditions were optimized and efficiencies were checked with a dilution series (5 points, 10-fold dilutions). Three replicates of each sample were used and five replicates of an inter-run calibrator (equal quantities of cDNA from all samples combined) were included in each qPCR run. CFX96 C1000 Touch Thermal Cycler (Bio-Rad) was used. A melt curve analysis was done at the end of each amplification reaction, and melt curves were checked to ensure that there was only a single PCR-product. ddPCR (IV) was done using BioRad QX200 system with EVA Green supermix, and QuantaSoft software for determining the concentration of the target (copies/µl) from the negative and positive droplets. See details of reaction mix and thermocycling protocol for qPCR in I, II, II and IV, and for ddPCR in IV.

2.6 Soil microcosm measurements (III)

CO_2 production was measured with a Calanus UniQuant carbon analyzer from air samples taken from the microcosm air space (eight measurements/simulated growing season) and used as a proxy for soil biological activity.NH_4-N concentration was measured from soil samples after the first and the second seasons, as well as from the original soil, using SFS-EN ISO 11732:2005 method, and used as a proxy for nutrient mineralization. The other parameters were measured after the first and the second growing seasons, and cocoon production, growth and mortality of the earthworms were used as measures of earthworm condition. Gene expression of five genes were used as a proxy for earthworm metabolic activity and expression of one gene involved in metal tolerance was used as a measure of physiological response of the earthworms to Cu contamination.

2.7 Experimental setups, data handling and statistical analyses

2.7.1. Metal exposure experiment (I)

Expression of metallothionein (MT) was measured from *D. octaedra* earthworms collected from populations with different metal exposure histories, and the possibility of time- or dose-response in metallothionein expression was tested among individuals from the two populations when exposed to three concentrations of Cu or Zn (low, medium and high), as well as control without metal contamination, for 7, 14 or 28 days. Expression of MT was also compared to metal body burden of the earthworms.

ANOVA and Pearson product-moment correlation (or Kruskal-Wallis and Spearman's rank-order correlation when assumptions of a normal distribution or equal variances were not met) were used in the analyses (see details in I).

2.7.2. Variation in gene expression in clones (II)

qPCR was used to examine variation in gene expression both within and among genotypes in two generations of earthworms. The experiment was begun using five individuals per genotype, from six different genotypes, three originating from Jyväskylä (uncontaminated site) and three from Harjavalta (metal contaminated site). This was the parent generation, and the earthworms were exposed to the contaminated soil for two months. The cocoons the parents produced were collected, placed on moist paper towel in lidded petri dishes and after hatching the young earthworms were also reared individually in the Cu contaminated (100 mg kg^{-1} dry mass) soil for two months. Offspring generation consisted of five offspring per parent, from three parents per genotype (only two of the six genotypes, other cocoons failed to hatch due to molding).

The main study question was how much variation in gene expression exists between the different groups (within genotypes vs. over all genotypes), and so the normalized Cq-values were used to calculate estimates of variation within the groups. The estimate of variation (EV) was calculated from the sample variance adjusted with the corresponding degrees of freedom for each group. Differences in the estimates of variation (EV) between the different groups (within individuals, within families, within genotypes, and over all genotypes) were compared, as well as differences in variation between different genotypes, families and populations, and between parents and offspring. Repeated measures ANOVA, Bartlett test, Wilcoxon Signed Ranks test and Mann-Whitney test were used.

In addition, the levels of gene expression (not just variation) were compared using normalized relative quantity (RQ) values. Comparisons were done between offspring of the same genotype, between genotypes, between populations and between offspring and parents of the same genotype using Mann-Whitney and Kruskal-Wallis tests (see details in II).

2.7.3. Effect of genetic diversity on decomposition processes (III)

The effect of *D. octaedra* genetic diversity on soil decomposition activity was examined with a year-long microcosm experiment simulating two growing seasons ("years") in which the microcosms contained *D. octaedra* with either low diversity (clones) or high diversity (different genotypes). Three different cultures (clone families) were used, and two of the clone families had the same multi-locus genotype based on genotyping with five microsatellite markers. Four or six replicates of the low diversity treatments were established (six replicates when using the clone family that differed from the other two). For the high diversity treatment three different genotype-compositions were used and there were four replicates of each. The experiment was done in different conditions: uncontaminated soil or moderately Cu-contaminated soil, and low or high diversity levels of other decomposer animals. Microcosms were incubated in a climate chamber with changing temperature to mimic different seasons (two "years"). After the first summer, half of the microcosms were destructively sampled and a second year was continued with the earthworms from the remaining microcosms, with new soil and new individuals of other decomposer species (1 *Dendrodrilus rubidus*, 10 individuals of *Cognettia sphagnetorum* and samples of microarthropods).

The main focus was to test the differences between low (LD) and high genetic diversity (HD) treatments, and it was tested in general (all conditions combined) and separately for the different conditions, and overall (combining the data from both years) and for the first and the second year separately. Differences between LD families, between HD and the two genotypes in LD separately, between genotypes, between uncontaminated and contaminated soil and between low and high diversity of other decomposer animals were also tested. One-way ANOVA or Kruskal-Wallis tests were used (see details in III).

2.7.4. Using droplet digital PCR technique for gene expression measurements (IV)

Repeatability and reproducibility of the ddPCR technique for gene expression measurement were tested by comparing the datasets and results produced by the same person twice at different times and by another person for the expression of two genes in the earthworm *D. octaedra* (three datasets compared). It was also tested whether the two methods, ddPCR and the traditionally used qPCR, produce consistent results when comparing expression of nine genes from earthworms originating from two different populations. In addition, effect of different RNA extraction methods on the consistency of the ddPCR results was studied. Reference genes could not be used for normalizing the gene expression data from ddPCR, so NORMA-Gene normalization was used for both ddPCR data and qPCR data, and also results from non-normalized data were compared. Difference between the three datasets for the repeatability and reproducibility test, and difference in gene expression between the two populations were tested with One-way ANOVA.

2.7.5. Raw data handling and general statistics (I, II, III, IV)

For the gene expression measurements, raw data from the qPCR runs were first analyzed with qBase+ (Biogazelle) to adjust results for the different reaction efficiencies and to perform inter-run calibration (Hellemans et al 2007). qBase+ was also used to check the stability of the reference gene expression for both qPCR and ddPCR data, and to calculate normalized relative quantities of the target gene, when the reference genes were stable (I, IV). qBase calculates the normalized relative quantities from the differences in cycle quantification values (Cq) between the target and the geometric mean of the reference genes (minimum of two reference genes), while taking the efficiencies of the PCR reactions into account. NORMA-Gene, a data driven normalization method (Heckmann *et al.* 2011), was used to normalize the relative quantities (qPCR) or concentration (ddPCR) when reference genes were not stable (II, III, IV). Data were checked for conformity to a normal distribution using a Kolmogorov-Smirnov test or Shapiro-Wilk test, and for homogeneity of variances with Levene's test, and when necessary, the data were log-transformed. When required after ANOVA tests (see below), the LSD post-hoc test was used for pairwise comparisons. When the assumptions of a normal distribution or equal variances were not met even after data transformation, non-parametric methods were used. All statistical analyses were carried out using IBM Statistics SPSS 20 or in Excel.

3 RESULTS AND DISCUSSION

3.1 Metallothionein gene expression differs in earthworm populations with different exposure history (I)

In my study, expression of metallothionein was higher in earthworms from populations with metal exposure history than in those from populations without metal exposure history, supporting my hypothesis of higher expression in earthworms originating from the contaminated site. When exposed to three different concentrations of copper or zinc for 7, 14 or 28 days, earthworms from the contaminated site had stable, high metallothionein expression, unaffected by exposure time or exposure concentration. In earthworms from the uncontaminated site the response was slow, expression increasing with exposure time. The difference in the expression response could be due to adaptation or acclimatization, since both strategies can be used by organisms to persist in metal contaminated environments (e.g. Aziz *et al.* 1999, Langdon *et al.* 2003, Kozloc and Zvereva 2011, Pauwels *et al.* 2013). Changes in expression of metallothioniens (e.g. over-expression and rapid expression response) are associated with adaptation in some soil invertebrates, e.g. the collembolan *Orchesella cincta* (Janssens *et al.* 2009, Roelofs *et al.* 2008, 2009) and the earthworm *Aporrectodea tuberculata* (Lukkari *et al.* 2004a). Since adaptation to copper has been demonstrated in my species *D. octaedra* before (Fisker *et al.* 2011a, 2011b, 2013), adaptation is likely to explain at least partly the higher (both field and exposure experiment) and stable (exposure experiment) expression of metallothionein in earthworms from the contaminated site. However, to be able to say with confidence that it was adaptation, F1 and preferably also F2 generation earthworms would have had to been tested to confirm genetic differences as the cause for the different responses. In the earthworms from the uncontaminated site, the upregulation of metallothionein was clearest after 28 days of exposure, suggesting that a longer exposure period improves the chances of observing the acclimatization response. The slow response might mean that earthworms without

metal contamination history cannot cope with metal contamination as well as earthworms with contamination history.

A consistent dose-response of expression was not found in either population, which could mean that the metals we tested, Cu and Zn, are not as efficient inducers of this isoform of metallothionein as e.g. cadmium (Cd). Cu and Zn are essential metals, and their uptake, export and intracellular compartmentalization are regulated in different ways (Valko *et al.* 2005), whereas the nonessential cadmium (Cd) is mostly sequestered with the help of metallothioneins (Sturzenbaum *et al.* 2004). A dose response is often found between metallothioneins and Cd (Marino *et al.* 1998, Swain *et al.* 2004, Spurgeon *et al.* 2004, 2005b Galay Burgos *et al.* 2005, Timmermans *et al.* 2005, Brulle *et al.* 2007, Brulle *et al.* 2011) but clear dose-responses are often not found for the essential metals Cu or Zn, with some exceptions (Spurgeon *et al.* 2005b, Brulle *et al.* 2007, Bundy *et al.* 2008). Although a dose-response was not found in my study, the expression of metallothionein was still affected by Cu and Zn exposure, as evidenced by the increasing expression in earthworms from the uncontaminated site with increasing exposure time. This suggests that metallothionein is involved also in regulation of Cu and Zn, and thus measuring the expression of metallothionein is relevant in exposure studies with these metals as well.

For the earthworms from the contaminated site, another reason for the lack of dose-response could be potential adaptation. Adaptation can lead to over-expression of stress-response genes (Roelofs *et al.* 2008), which could mean that individuals from adapted populations might show no response or only a small response to increasing concentrations of metals (Pauwels *et al.* 2013). This is supported in my results by metallothionein expression being higher in earthworms from the contaminated site even when they were not exposed to any added metal, and the similar high levels of expression in all exposure concentrations. On the other hand, adaptation does not necessarily mean that there cannot be a dose response. Dose-responses are known for individuals originating from populations with exposure history e.g. in *Aporrectodea tuberculata*, for which concentration of metallothionein increased with increasing soil metal concentration, especially in the first week of exposure (Lukkari *et al.* 2004a). Thus, it could be context dependent whether the inducibility of a stress response is lost in populations with exposure history.

High variation among individuals in metallothionein expression was found, both in samples from the field and in the exposure experiment. Heterogeneous distribution of metals in the soil (Salminen and Haimi 1999) and avoidance behavior of *D. octaedra* (Lukkari and Haimi 2005) could lead to individual variation in exposure to metals and consequently to variation in metallothionein expression in the contaminated field conditions. But, it does not explain the variation between individuals from the uncontaminated site (no avoidance needed) and variation in the exposure experiment (since avoidance was not possible), suggesting that there are other causes behind the variation. High genetic diversity (Simonsen *et al.* 2008, Knott and Haimi 2010) and polyploidy of *D. octaedra* (Omodeo 1955, Hongell and Terhivuo 1989) could also ex-

plain the high inter-individual variation in metallothionein expression. Genotypes can have large differences in their response (Nota *et al.* 2013), and differences in ploidy could lead to some individuals having more copies of the metallothionein gene than others, which could lead to differences in expression of metallothioneins. The high inter-individual variation found in this study was one of the motivations for taking a closer look into variation in gene expression within and between genotypes (II).

3.2 Variation in gene expression within clones of the earthworm *Dendrobaena octaedra* (II)

Microsatellite markers were used to verify that the earthworm *Dendrobaena octaedra* produces clonal offspring as expected, because *D. octaedra* is known to reproduce through apomictic parthenogenesis (Omodeo 1955, Hongell and Terhivuo 1989, Terhivuo and Saura 1990). Clonality was checked because in an allozyme study by Simonsen and Holmstrup (2008) some variation was found between offspring and their parent. The microsatellite markers showed that offspring are clones of their parent, but the number of markers was only five, and since one of them was monomorphic, the number of useful markers was four. From the different cultures, many different genotypes (16) were found with the four microsatellite markers, thus the markers were able to differentiate between individuals. The markers were useful for choosing different genotypes to use in the study about effect of genetic diversity on ecosystem functioning (III). However, the low number of markers does leave open the possibility that there could still be undetected genetic differences between the individuals determined to have the same genotype.

After verifying clonal reproduction, expression of genes related to stress response and metabolism was measured in individuals from two generations, parents and offspring, and variation in gene expression both within and among clonal genotypes was determined. The results showed that gene expression can differ between genetically identical individuals exposed to the same conditions, even as much as between different genotypes. Other studies have shown that there is high variation in gene expression (Levine and Tjian 2003, Lopez-Maury *et al.* 2008, Viney and Reece 2013, Rosa *et al.* 2014, Rue and Arias 2015), and in light of that, it might be unrealistic to assume that genetically identical individuals exposed to the same conditions will express genes exactly similarly. I hypothesized that there would be more variation in gene expression between genotypes than within genotypes, but the hypothesis was supported only partly. In the parent generation there was significant variation in gene expression among individuals sharing the same genotype, but still genetically identical individuals were more similar in their gene expression than were individuals that were genetically different. However, in the offspring dataset, and also in the parent dataset when only a subset of five genes was analyzed, the variation in gene

expression within genotypes was as high as the variation over all genotypes. Variation was high even within families (among offspring that were from the same parent).

Gene expression variation can help organisms cope in changing environments through plastic responses, and in the long-term through adaptation to the changed conditions (Levine and Tjian 2003, Lopez-Maury *et al.* 2008, Raj and van Oudernaarden 2008, Viney and Reece 2013). Genes related to stress have been found to show more variation than other genes involved in basic cell maintenance functions (Blake *et al.* 2006). In my study, the three stress related genes, HSP40, HSP70, and MT showed high variation within genotypes, but so did many of the genes related to metabolism. Studies on cell-to-cell variation in gene expression provide information on some possible mechanisms behind gene expression variation, e.g. there can be transcriptional bursts in gene expression, when genes switch between transcriptional activity and inactivity (Raj and van Oudernaarden 2008). Epigenetic regulation could also be behind the variation found in my study. Kille and colleagues (2013) found high methylation in the earthworm *Lumbricus rubellus* and, for one of the two lineages they studied, the methylation correlated with soil arsenic concentrations. However, whether DNA methylation occurs in *D. octaedra* and affects gene expression in this species is not known. Determining the mechanisms behind the gene expression variation among individuals sharing the same genotype was beyond the scope of this study, but is an interesting topic for future studies. Finding out what causes the variation could make it easier to take the variation into consideration in comparative gene expression studies, e.g. by using specific tissues only or by determining the level of methylation in the individuals used.

One explanation for finding variation in gene expression within a single clonal genotype could be that there is unidentified genetic variation within the sample and that the individuals are not really clones. In the collembolan *Folsomia candida,* two different genotypes, originally assumed to be clones, were found in one strain (Nota *et al.* 2013). In this study, there is a possibility that mutations have occurred during the culturing time and that the individuals are genetically different despite originating from a single parent. However, it is unlikely, since the culturing times lasted only 1-2 generations, and genomic mutation rates are often not very high, e.g. 0.0026 per gamete per generation for *Caenorhabditis elegans* (Keightley and Caballero 1997). Changes in the chromosome ploidy, including aneuploidy has been found in *D. octaedra* (Omodeo 1955, Hongell and Terhivuo 1989), and it might be more likely explanation for the differences than mutation, since changes in the number of chromosomes can lead to large differences between individuals. There is also a possibility of undetected meiosis, as was recently found for the bdelloid rotifers in the clade *Macrotrachela quadricornifera* previously thought to be entirely asexual (Signorovitch *et al.* 2015). Genotyping with the microsatellite markers confirmed that offspring had the same multi-locus genotype as their parent and each other, but the number of markers limits how strong conclusions can be drawn: genetic differences might have been overlooked, and using more genetic markers could

have made stronger conclusions possible. For example, ten microsatellite markers were developed for the earthworm *Lumbricus terrestris* to be used in parental analyses and population genetic studies (Velavan *et al.* 2007).

The results also indicate that the degree of variation can depend on the gene studied as well as the genotype. Some genes showed more variation than others, and there was difference in variation between different genotypes. In the offspring dataset, one of the studied genotypes showed significantly higher variation than the other. Gene expression levels of most genes were significantly different between the genotypes in both parents and offspring, and in one gene (ChymInh) even between offspring families sharing a genotype. In a study by Nota and colleagues (2013), two different genotypes of collembolan *Folsomia candida* had highly different response to cadmium exposure, one being sensitive to it and one tolerant. In the water flea *Daphnia* different genotypes within two ecotypes (originating from a lake or from a pond) differed in their gene expression response to different resource environments (Dudycha *et al.* 2012). Different asexual lineages of *Dendrobaena octaedra* in natural populations might also differ in responsiveness to environmental conditions. This suggests that variation within genotypes could affect the results of comparative studies of gene expression phenotypes: since some genotypes show more variation than others, results could be biased by what genotypes happen to be included in the sample, or the results could be specific only to the genotype used, when monoclonal lines are used.

Some variation in gene expression measurements is unavoidable due to technical factors. Different RNA extraction methods and differing amounts of inhibitors, efficiency of cDNA synthesis, PCR efficiency of the primers used and pipetting accuracy can cause variation in the sample quality and gene expression measurements. The MIQE Guidelines (Minimum Information for Publication of Quantitative Real-Time PCR) give valuable instruction for minimizing variation and for producing data that is comparable between different laboratories and people (Bustin *et al.* 2009). The guidelines include instructions on e.g. checking RNA concentration, checking efficiencies of the primers and normalizing the expression data against more than one reference genes or with some other methods (Bustin *et al.* 2009). I minimized the effect of technical factors e.g. by measuring the concentration of RNA and equalizing it in all samples before cDNA synthesis, by using three technical replicates in qPCR to diminish the effect of pipetting errors, by having an inter-run calibrator to check for variation between different runs and by normalizing the expression data with NORMA-Gene (Heckmann *et al.* 2011). The effect of technical variation is thus likely to be small in my study but cannot completely be excluded as a source for some of the variation. Another technique for measuring gene expression, the droplet digital PCR, is supposed to suffer less from the technical issues (Huggett *et al.* 2013), which is why I decided to test that technique also with my samples (IV).

3.3 Genetic diversity of *D. octaedra* affects ecosystem functioning (III)

I found some indications that higher genetic diversity of *D. octaedra,* originating from a population with metal contamination history, has a positive effect on decomposition processes and that high genetic diversity of the earthworms is especially beneficial in stressful conditions, which supported my hypothesis. In my microcosm experiment, NH_4-N concentration was higher in the high diversity treatment in contaminated soil. Earthworms in the high diversity treatments seemed to thrive (higher biomass) in contaminated soil, in uncontaminated soil they suffered from higher mortality and did not increase microbial activity (lower CO_2 production). Biodiversity and ecosystem functioning are often positively correlated (Gravel *et al.* 2011, Hooper *et al.* 2012, Harrison *et al.* 2014, Duncan *et al.* 2015, Evans 2016) and my results showed that the positive effect of diversity can to some extent apply also at the genetic level and in terrestrial ecosystems. However, the support for the benefits of higher genetic diversity was not very strong, since there was no difference between low genetic diversity and high genetic diversity in many of the parameters studied, or the difference was significant for only one of the two growing seasons. The results of my comparisons between low and high genetic diversity treatments are summarized in Table 1.

TABLE 1. Differences between low genetic diversity (LD) and high genetic diversity (HD) treatments in the different experimental conditions: uncontaminated or Cu contaminated soil, low (indicated by minus-sign) or high (indicated by plus-sign) species diversity of other decomposer animals. Zero means no difference between the genetic diversity treatments, LD means the parameter measured was higher in low diversity and HD means it was higher in high diversity treatment. Results are given for the two growing seasons ("1.year and 2.year") separately.

	Uncontaminated-		Uncontaminated+		Contaminated-		Contaminated+	
	1.year	2.year	1.year	2.year	1.year	2.year	1.year	2.year
NH_4-N conc.	0	0	0	0	0	0	HD	0
CO_2 prod.	0	0	LD	0	0	0	0	0
Biomass	0	0	0	0	0	0	0	HD
Cocoon prod.	0	0	0	0	0	0	0	0
Mortality	HD	0	0	0	0	0	0	0
AkRed	0	0	0	0	0	LD	0	0
ChymInh	HD	HD	0	0	HD	0	0	0
DualPhos	0	0	0	LD	0	HD	0	0
GlucReg	0	0	0	0	0	0	0	0
PanAmy	0	0	0	0	0	0	0	0
MT	0	0	LD	0	0	0	0	0

There was higher CO_2-production and earthworm growth in contaminated soil than in uncontaminated soil in both treatments, suggesting that earthworms were coping well in contaminated soil. Li and colleagues (2010) found that in algal communities species diversity was important for productivity in cadmium-polluted conditions, and species tolerant to Cd were doing especially well. The earthworms in my study originated from a population with decades long history with metal contamination (Salemaa 2003), and adaptation of *D. octaedra* to these conditions is likely (I). Copper contamination at a moderate level can have positive effects on earthworms, e.g. on growth and reproduction of *Aporrectodea tuberculata* (Lukkari *et al.* 2005) and *D. octaedra* (Bindesbol *et al.* 2007, Fisker *et al.* 2011). Reasons for that are unknown but it could possibly be because copper is an essential metal and soil without added metal might have a lower than optimal concentration of Cu.

Because *D. octaedra* is not alone in contributing to decomposition, the effect of low or high species diversity of other decomposer animals on the relationship between genetic diversity of *D. octaedra* and decomposition processes was also studied. Most of the differences between low and high genetic diversity treatments only showed statistically significant differences when there was a high diversity of other decomposer animals (usually only in one of the growing seasons). Specifically there was higher NH_4-N concentration in HD in contaminated soil with high diversity of other decomposer animals, indicating that there are some positive effects of higher genetic diversity together with high species diversity. However there was also higher CO_2 -production in LD in uncontaminated soil with high diversity of other decomposer animals, which is contrary to the positive effect of higher genetic diversity. Finding these differences between low and high genetic diversity was in contrast to my hypothesis that high diversity of other decomposer animals might mask the differences between low and high genetic diversity treatments due to more functional groups contributing to decomposition.

However, at the end of the experiment, the only positive effect of having higher species diversity of the other decomposer animals was higher biomass of the earthworms. More functional groups can lead to more efficient decomposition processes (Laakso and Setälä 1999, Ekschmitt *et al.* 2001, Cragg and Bartgett 2001, Heemsbergen *et al.* 2004), and so I expected additional species to increase decomposition efficiency, but it did not. This supports conclusions made by some researchers that in soil the presence of key-decomposers is more important for ecosystem functioning than is the decomposer diversity (Cragg and Bardgett 2001, Heemsbergen *et al.* 2004). However, I studied decomposition processes in general and could not isolate specifically the impact of *D. octaedra* on decomposition activity. Also, detecting an impact of the higher diversity of the other decomposer animals might have been difficult since the microcosms had limited structural diversity (homogeneous soil) and few decomposer species. Moreover, there was high mortality of the added earthworm species, *D. rubidus,* which is another key-decomposer possibly with slightly different feeding strategy than *D. octaedra.* Had the *D. rubidus* lived, there might have been

more pronounced differences between microcosms with low or high diversity of the other decomposer animal species.

For the gene expression measurements, genes expected to be involved in metabolism were selected, and also a gene for the metal-tolerance protein metallothionein (MT) was included. MT has been linked to adaptation of *D. octaedra* to metal contamination (Fisker *et al.* 2013, I). The specific function of MT and the functions of the other gene products have not been experimentally confirmed in *D. octaedra.* I had to rely on the original GO-terms assigned to the transcriptome-sequences, thus I can only speculate about functional responses when examining the results of my gene expression comparisons. Overall expression of MT was higher in metal contaminated conditions, which was expected. For the genetic diversity treatments, the only significant difference between low and high genetic diversity treatments was that in uncontaminated soil with high species diversity, expression of MT was higher in *D. octaedra* with low genetic diversity than high diversity. This difference is probably not related to the function of MT in metal-tolerance, since the difference was found only in the uncontaminated soil. In the study about expression of MT between earthworms with different exposure histories (I), I found that there was high variation among individuals of *D. octaedra* in expression of MT, both in contaminated and uncontaminated soil. Genotypes in the low diversity treatment could, by chance, be genotypes that express MT at a higher level in uncontaminated soil, and the genotypes in high diversity those that express MT at a lower level. Or, the high diversity treatment could have had genotypes expressing MT at both low and high levels, leading to overall higher expression in the low diversity treatment. Some differences in expression of the other genes were also found between the low and high genetic diversity treatments. Chymotrypsin inhibitor was most consistent in showing higher expression in the high genetic diversity treatment than in the low diversity treatment. For the other metabolic genes studied, only a few differences between the low and high genetic diversity treatments were observed, and sometimes only under certain conditions, preventing any generalization of the gene expression patterns. Additional studies into the function of these genes in *D. octaedra* are needed before conclusions on what the differences mean at the functional level can be made.

An interesting result was that the specific genotype or lineage of the earthworms seems to explain some differences between the treatments instead of genetic diversity *per se.* For many of the parameters studied, the effect of the diversity treatment was dependent on whether the high genetic diversity group was compared to one (LD1) or the other (LD2 and LD3) of the genotypes in the low diversity treatments. For example, I observed higher NH_4-N concentration in the low diversity treatment when high diversity was compared to LD1, but it was higher in the high diversity treatment when high diversity was compared to LD2 and LD3. Also, more CO_2 was produced in the low genetic diversity treatment when high diversity was compared to LD2 and LD3, but there was no difference when it was compared to LD1. In gene expression, the genotype used in the low diversity treatment had a large impact on the results: no differences

were found in gene expression between high diversity and low genetic diversity treatments when high diversity was compared to LD2 and LD3, but differences were evident when comparing the high diversity treatments to LD1. In my earlier study I found high variation both between and within genotypes (II). The results of this study (III) showing differing responses depending on genotype further support the notion that a large sample should be considered in gene expression comparisons since results from one genotype might not be the same as from another.

In addition, how the genotypes responded to the different conditions (uncontaminated or contaminated) differed in microcosms with high diversity of other decomposer animals. Genotypes LD1 and LD2 had a similar response in NH_4-N concentration, CO_2 production and gene expression to contaminated and uncontaminated conditions despite being different genotypes. In contrast, the response of genotype LD3 differed. This could suggest that LD2 and LD3, which were the same genotype according to the five microsatellite markers described earlier (II) but were reared from different founding individuals, are not, in fact, genetically identical. Thus, the need to verify that individuals assumed to be clones truly are clones and the need for more information on the mechanisms causing variation in presumed genetically identical individuals are also supported by this study (III).

3.4 Using droplet digital PCR for gene expression measurements

The droplet digital PCR (ddPCR) technique was highly repeatable and reproducible for gene expression measurements in *D. octaedra*. The original dataset, the dataset repeated by the same person and a dataset produced by a different person did not differ from each other. The datasets also gave the same results for a population comparison (difference between populations in expression of ChymInh, no difference in expression of Leuc). However, when comparing the two techniques, ddPCR and qPCR, there were some inconsistencies in the results of the population comparison. ddPCR is more sensitive, able to detect smaller differences in expression (Huggett *et al.* 2013), but in my results more differences between the populations were found using qPCR than with ddPCR. Reliability of qPCR measurements has been studied before, and although it is generally considered to be simple and reliable method for gene expression measurement, there are many steps in the process in which technical variation can be introduced, from the RNA extraction methods through cDNA synthesis to normalization method used, making replication of results challenging (Sanders *et al.* 2014). Thus, the differences between populations found with qPCR that were not found with ddPCR could be due to technical factors leading to statistical differences without actual biological differences. Moreover, the inconsistencies could also be due to the normalization method used.

Gene expression measurements need to be normalized to account for the non-biological variation, caused by variation in sample quality and efficiency of

the cDNA synthesis (Hugget *et al.* 2005, 2013, Bustin *et al.* 2009, Kozera and Rapacz 2013). Normalization is often done with so-called reference genes with stable expression. The two potential reference genes I tested could not be used with the ddPCR technique. These genes are expressed at a much higher level than the target genes and in ddPCR all droplets were positive droplets. Diluting the samples helped resolved the problem, but there was too much variation in the expression of the reference genes, either due to the dilution steps or because the ddPCR was more sensitive to detecting the variation than was qPCR. Thus, reference gene normalization was not possible for ddPCR, and another normalization method, NORMA-Gene was used for both datasets. The inconsistencies between population comparisons with qPCR and ddPCR showed in those made with the NORMA-Gene normalized data. When I analyzed the non-normalized data the population comparisons were consistent. Not normalizing the gene expression is not an option in any real comparisons of gene expression response since normalization is needed to minimize the non-biological variation (Hugget *et al.* 2005, Kozera and Rapacz 2013), and it was done here only to compare the techniques, not to interpret the differences between the populations in gene expression. More significant differences between populations were found with NORMA-Gene normalized data both with ddPCR and especially with qPCR, and in qPCR also with reference gene normalized data, than with non-normalized data. This highlights the large effect that the normalization method can have on the results, which deserves more attention in future studies. The high repeatability and reproducibility of ddPCR make it a good technique for gene expression measurements, and results from ddPCR that are consistent with results from the qPCR technique might be possible if a normalization method suited for both techniques was used.

4 CONCLUSIONS

The aim of the thesis was to study the effect of genetic diversity within a key decomposer (here the earthworm *Dendrobaena octaedra* as an example) on decomposition processes in metal contaminated conditions, including the effect of population metal exposure history and possible variation in gene expression. The observations by some researchers that biodiversity might be more important for ecosystem function in metal contaminated conditions than in uncontaminated conditions (Farina *et al.* 2003, Li *et al.* 2010) motivated me to look into the relationship at the genetic level. Some support for a positive effect of genetic diversity on decomposition in contaminated soil was found in my study, but the conclusions were dependent upon which genotypes were included in the experiment. The results indicated that even when there is low genetic diversity, ecosystem functions can be maintained as well as in the presence of high genetic diversity, depending on the genotypes present (III). This corresponds to the predictions that in soil, there is redundancy in species diversity, and the presence of key species is more important for decomposition (e.g. Ekschmitt *et al.* 2001). My results show that this is true at the genetic level also.

D. octaedra is expected to reproduce through apomictic parthenogenesis and this was supported in my study. The microsatellites I used offer an additional way of assessing clonality and to also distinguish different genotypes. The number of functional markers was small (four), which leaves open the possibility that there still is some undetected genetic differences between individuals I assumed to be clones. The undetected variation might explain the high variation among genotypes in gene expression (II) and the differing responses of the earthworm families to different conditions in the microcosm experiment (III). In the future, it would be important to study more how much variation in gene expression exists in truly genetically identical individuals and how much might be due to non-genetic variation (epigenetics). This could be especially important in ecotoxicological studies, in which clonal lines are often used to study the response of test organisms to contaminants, and decisions about the environmental issues are made based on the results.

Because there is variation in gene expression it is advisable to use many different genotypes to get a reliable average of a population's gene expression response. My results (II and III) show that conflicting conclusions could be made depending on which genotypes are chosen for a particular study when there is small sample size. Using more genotypes could reveal more about how much variation there can be within and between genotypes and how different kinds of genotypes affect the decomposition. Having many different kinds of genotypes with varying abilities to participate in decomposition could help earthworms cope in metal contaminated conditions through complementation, and through increased adaptation potential.

The high repeatability and reproducibility of droplet digital PCR (IV) makes it a good option for gene expression measurements, but in my study there was no advantage of ddPCR over qPCR. For gene expression measurements absolute quantification is not necessarily needed and ddPCR results might be difficult to compare to other studies using relative quantities. The ddPCR could be better for measurements, in which greater precision is needed and absolute quantitation is required, for example, to determine copy number variation. My study species, *D. octaedra* is hexaploid, but there can be variation in the ploidy, including aneuploidy. It would be interesting to see if there is variation in the copy number of e.g. metallothionein and how that variation affects the gene expression.

D. octaedra is a good study species when investigating variation in gene expression and the effects of genetic diversity on decomposition in metal contaminated condition. Because of its high genetic diversity, there are potentially many different genotypes with functional differences. Even when using only two different genotypes in low genetic diversity treatment, I found differences in responses to metal contamination in comparison to the high genetic diversity treatment (III). It is suitable for future studies as well, e.g. for an investigation into copy number variation, or for studying effects of genetic diversity on decomposition more broadly than here. However, the long generation time of *D. octaedra* requires time for culturing, if clones are needed.

This thesis gives new information about the diversity-functioning relationship in terrestrial ecosystems: genetic diversity and gene expression variation in a key decomposer affects decomposition processes; but more questions were also raised. For example, I found that exposure history has an effect on the earthworms' gene expression response to metals (I), so would the effect of genetic diversity on decomposition be different if earthworms without metal exposure history had been studied? How would a non-essential metal, such as cadmium, affect the biological response and the relationship between genetic diversity and ecosystem functioning? How are the differences in gene expression among individuals affecting the population at the functional level? My thesis will encourage future efforts on these important questions.

Acknowledgements

First, I would like to thank my two wonderful supervisors, Emily Knott and Jari Haimi. Emily, you have been a huge part of my scientific endeavours since my Bachelor thesis. You have always been so supportive and cheerful that I have felt beyond lucky to have had you as a supervisor all these years. Jari, the first time I met you, I had just started at the university and you were teaching a basic course. Your enthusiasm was contagious even then, and during these four years, your support and positive outlook have helped me tremendously. Both of you have always been so available and approachable, so willing to advice and inspire me that I never had to feel like I was alone in my research. Your guidance and encouragement have helped me develop as a scientist, and made my PhD work a joy to do.

Jenni Kesäniemi, thank you for being a coauthor in one of the papers, but even more than that I want to thank you for being a great supervisor during my Bachelor and Master theses, and for being fun and supportive roommate, to whom I could always turn to when I had questions or concerns. I would also like to thank my other co-authors: Ari Väisänen, thank you for teaching me how to extract metals from the samples and being part of my first PhD paper. Harri Högmander, thank you for your help with the tricky statistics and co-authoring in the second paper. I would also like to thank Annaliisa Kankainen for the help with those statistics. Thank you, Maaria Kankare for co-operation, and Martin Holmstrup for sharing the draft transcriptome of *D. octaedra*, which was essential for this work, and both of you for being in my follow-up group. It was very nice and helpful to get feedback from you in those meetings. I also want to thank the pre-examiners for their time and effort in examining my thesis.

My work was funded by Maj and Tor Nessling Foundation and University of Jyväskylä, I very much appreciate the financial support which made this research possible. I'm also grateful for the opportunities to attend conferences, like the SETAC meeting in Switzerland, EMPSEB meeting in Scotland, Nordic Oikos and Molecular Ecology meetings here in Finland and the BIOINT meeting in Lammi. I am also very grateful for the two weeks I got to spend in Konnevesi finishing my thesis.

I want to thank Martin Chilman for the help with the earthworms and in the lab. You got some of the more boring work, and did it enthusiastically and without complaint for many months, and it was a huge help to me. I also want to thank student Tarja Työläjärvi for doing your Bachelor thesis in our little group and working hard on it. Thank you, Mustapha Boucelham, for the help with the soil sample processing. I also want to thank the lab people for always being willing to advice and help, especially Elina Virtanen but also Sari Viinikainen, Sami Kyröläinen and Juho Niva.

People in our department have been great coworkers. The courses I have taken have been very helpful, thanks to the inspiring and accomplished teachers, and other PhD students and staff have been fun to work with. Thank you to all my room mates over the years, you have all been very nice to share a room

with. Sauna and Support has been great, thank you all who have participated in them and especially the people who have organized them. Outside work I have gotten to relax and enjoy the company of my colleagues at the poker-nights, thank you all who have been there and especially Steve for organizing them.

I want to thank the coffee ring, Anne, Hannele, Jenni, Nina and Venera, for the much needed breaks and nice conversations and support. Also the evolutionary genetics meetings and celebrations for accepted papers have been great, I want to thank Anneli Hoikkala, and all the people who have organized and participated in those.

I am so lucky to know and be friends with some of the most amazing people. Sanna, we don't see each other as much as I would like since you don't live in Jyväskylä anymore, but just knowing that you, and Hannu, are always there for me gives me so much comfort. In recent years I have gotten so many great friends that it would be too long list to mention you all by name, but I want to thank you for welcoming me into your lives and let you know that I value our friendships beyond words.

I want to thank my mom. For raising me alone, for always supporting my decisions and for setting the example of a truly good person. If I can ever be even half as strong and half as kind as you are, I consider myself to be very lucky. Kiitos, äiti. I also want thank my little brother Matias. It has been great to watch you grow from that tiny little baby into the kind and funny young man you are now, and I'm so happy to call you my friend as well as brother. I also have two wonderful big sisters. Satu, we might not be biologically sisters, but we are in every way that matters. Thank you for being in my life, and bringing Pete and Milla into it also. Marjut, I have always looked up to you and Saska, and your adventurous nature. I also want thank my niece Kiia and nephew Tuukka. Your energy is something else, and it has been great to watch you grow.

Then, finally, I want to thank my love, Paavo. You have been so supportive and understanding throughout this project, and in the many years we have been together. Every joy is so much better when I get to share it with you, and every hardship so much easier to bear with you by my side. You and our lovely cats Haisuli, Mörkö and Vatti make a wonderful home for me, filled with love and laughter. Thank you.

YHTEENVETO (RÉSUMÉ IN FINNISH)

Geenien ilmentymisen vaihtelu metsälierolla ja lajin geneettisen diversiteetin vaikutus ekosysteemin toimintaan metallisaastuneessa maassa

Korkealla biodiversiteetillä on usein positiivinen vaikutus ekosysteemin toimintaan, eli prosesseihin, jotka liittyvät aineiden kiertokulkuun ja energian virtaukseen ekosysteemeissä. Biodiversiteetistä puhuttaessa keskitytään usein lajien diversiteettiin, mutta myös lajien sisäisellä geneettisellä diversiteetillä voi olla merkittävää vaikutusta ekologisiin prosesseihin. Geneettinen diversiteetti saattaa olla erityisen tärkeää dominoivilla tai toiminnallisesti merkittävillä lajeilla sekä ihmisen muuttamissa ympäristöissä, kuten metallisaastuneilla alueilla.

Lierot vaikuttavat merkittävästi maaperän rakenteeseen ja ravinteiden kiertoon. Monet lierolajit ovat hermafrodiitteja ja lisääntyvät risti- tai itsesiitoksella, mutta myös partenogeneettinen lisääntyminen on lieroilla yleistä. Geneettisen diversiteetin on havaittu vaihtelevan suuresti eri lierolajien ja sukulinjojen välillä. Tutkimuslajillani, mitoottisen partenogeneesin avulla lisääntyvällä metsälierolla (*Dendrobaena* octaedra), on korkea geneettinen diversiteetti.

Metalleja päätyy ympäristöön monenlaisessa ihmistoiminnassa, ja niillä on usein negatiivinen vaikutus eliöihin. Metallit sitoutuvat erityisesti orgaaniseen ainekseen ja kertyvät maaperän karikekerrokseen, josta ne poistuvat hyvin hitaasti. Monet lajit eivät menesty metallisaastuneessa maassa, joten metallit vähentävät lajidiversiteettiä, ja sen kautta vaikuttavat ekosysteemin toimintaan. Metallien vaikutuksesta geneettiseen diversiteettiin on saatu ristiriitaisia tutkimustuloksia, eikä metallien vaikutusta ekosysteemin toimintaan geneettisen diversiteetin muutosten kautta ei ole juurikaan tutkittu. Lieroja käytetään usein indikaattorina metallien ympäristövaikutuksista. Näkyvimpiä muutoksia, kuten lajien häviämisiä, tapahtuu yleensä vasta korkean ja/tai pitkäkestoisen metallikontaminaation seurauksena. Välittömämpiä metallien vaikutuksia voidaan tutkia esimerkiksi mittaamalla eliöiden metallitoleranssiin liittyvän metallotioniinin geenin ilmentymistä.

Geenien ilmentymisessä on paljon vaihtelua, mikä auttaa eliöitä mukautumaan ja sopeutumaan muuttuviin oloihin. Tämä voi kuitenkin tehdä geenien ilmentymisen käyttämisen eliöiden fenotyyppisenä vasteena haastavaksi ja vaikeuttaa tutkimustulosten tulkintaa. Usein vaihtelua pyritään tutkimuksissa vähentämään käyttämällä esimerkiksi kloonilinjoja. Mahdollinen ongelma kuitenkin on, että tulokset, joita saadaan yhdestä kloonilinjasta, eivät välttämättä pädekään toiseen linjaan. Klooneiksi oletettujen yksilöiden välillä voi myös olla huomaamatta jäänyttä geneettistä erilaisuutta. Geenien ilmentymisen tutkimisessa on myös teknisiä haasteita, ja perinteisesti käytetyssä kvantitatiivisessa polymeraasiketjureaktiossa (qPCR) on monia vaiheita, jotka voivat tuottaa eibiologista vaihtelua. Uudessa menetelmässä, droplet digital PCR (ddPCR), tekninen vaihtelu on vähäisempää. Molemmissa menetelmissä on tärkeää, että geenien ilmentymisen mittaukset normalisoidaan luotettavalla menetelmällä, kuten tasaisesti ilmentyvillä referenssi-geeneillä.

Väitöskirjatutkimukseni tavoitteena oli selvittää miten geneettinen diversiteetti vaikuttaa ekosysteemin toimintaan, sekä miten populaation metallialtistushistoria vaikuttaa geenien ilmentymisvasteeseen, ja kuinka paljon vaihtelua geenien ilmentymisessä on genotyyppien välillä ja niiden sisällä. Tähän tarkoitukseen metsäliero oli sopiva tutkimuslaji monestakin syystä. Laji lisääntyy mitoottisen partenogeneesin avulla, minkä ansiosta oli mahdollisuus tutkia sekä alhaista (yksilöt klooneja) että korkeaa geneettistä diversiteettiä (yksilöt eri genotyyppejä). Metsäliero on tärkeä hajottaja ja usein avainlaji metsämaaperässä, joten geneettisen diversiteetin vaikutusten tutkiminen tärkeään ekosysteemin prosessiin, hajotukseen, oli mahdollista. Lisäksi metsäliero pystyy elämään metallisaastuneessa maaperässä, joten sekä metallialtistushistorian että geneettisen diversiteetin vaikutusten tutkiminen metallisaastuneessa maaperässä oli mahdollista.

Ensin tutkin metsälieron vastetta metallikontaminaatioon ja populaation historian vaikutusta siihen. Metallitoleranssiin vaikuttavan proteiinin, metallotioniinin geenin ilmentymistä mitattiin yksilöistä, jotka oli kerätty populaatioista, joilla joko oli tai ei ollut metallialtistushistoriaa. Populaatioiden yksilöille tehtiin koe, jossa ne altistettiin kolmelle eri kuparin ja sinkin pitoisuudelle kolmen pituisiksi ajanjaksoiksi. Lieroilla, joilla oli metallialtistushistoria, metallotioniinin ilmentyminen oli korkeaa ja tasaista metallipitoisuudesta ja altistuksen kestosta riippumatta, kun taas lieroilla, joilla ei ollut altistushistoriaa, vaste oli hidas. Adaptaatio metallisaastuneisiin oloihin on todennäköinen selitys eroille.

Seuraavaksi varmensin, että metsälieron jälkeläiset todella ovat geneettisesti identtisiä vanhempansa kanssa, kuten mitoottisella partenogeneesillä lisääntyvällä lajilla kuuluisi olla. Aiemmassa allotsyymi-tutkimuksessa oli löydetty poikkeavuuksia jälkeläisten ja niiden vanhempien välillä. Genotyypitin jälkeläisiä ja niiden vanhempia viidellä mikrosatelliitti-markkerilla, ja jälkeläiset olivat klooneja vanhemmastaan, mikä tukee mitoottista partenogeneesiä tällä lajilla. Kuitenkin markkereiden vähyys jättää mahdollisuuden auki sille, että klooneiksi oletettujen yksilöiden välillä on geneettisiä eroja, jotka jäivät huomaamatta. Geenien ilmentymisessä voi olla paljon vaihtelua, joten tutkin myös kuinka paljon tätä vaihtelua on sekä genotyyppien välillä että niiden sisällä selvittääkseni vaihtelun mahdollisia vaikutuksia geenien ilmentymisen käyttämiseen vasteiden vertailussa. Geenien ilmentymisessä oli suurta vaihtelua genotyyppien välillä, ja vaihtelu genotyyppien sisällä oli joissain tapauksissa yhtä suurta. Nämä tulokset korostavat tarvetta ottaa genotyyppien sisäinen vaihtelu huomioon tutkimuksissa, joissa vertaillaan geenien ilmentymistä eri ryhmien välillä, esimerkiksi vasteena metallialtistukseen.

Geneettisen diversiteetin vaikutusta ekosysteemin toimintaan tutkin vuoden kestäneellä mikrokosmoskokeella. Mikrokosmoksissa oli joko alhainen (metsälierot samasta kloonista) tai korkea geneettinen diversiteetti (metsälierot eri genotyyppejä). Selvittääkseni metallikontaminaation ja muiden hajottajien läsnäolon vaikutusta geneettisen diversiteetin ja ekosysteemin toiminnan suhteeseen mikroskosmosten maa oli joko puhdasta tai kuparilla kontaminoitua, ja muita hajottaja-eläimiä oli koemaassa joko alhainen tai korkea lajidiversiteetti.

Kokeessa mitattiin eri parametrejä hajotustoimintaan ja lierojen metaboliaan ja kuntoon liittyen. Geneettisen diversiteetin positiivinen vaikutus hajotustoimintaan sai hieman tukea kokeeni tuloksista, erityisesti metallisaastuneessa maassa. Usein kuitenkin ero alhaisen ja korkean geneettisen diversiteetin välillä riippui siitä mihin klooniin korkeaa geneettistä diversiteettiä verrattiin. Tuloksista voi päätellä, että ekosysteemin toiminnan kannalta tärkeämpää ovat genotyyppien piirteet kuin varsinaisesti geneettinen diversiteetti.

Mittasin geenien ilmentymistä qPCR-menetelmällä, ja vaikka sen yleisesti ottaen katsotaan olevan tehokas ja luotettava menetelmä, on siinä havaittu myös joitain ongelmia, kuten alukkeiden PCR-tehokkuuden ja cDNA synteesin tehokkuuden vaikutusta tuloksiin. Geenien ilmentymisen mittaaminen oli niin tärkeässä osassa muissa tutkimuksissani, että oli aiheellista tutkia myös miten uusi, vähemmän teknisestä vaihtelusta kärsivä menetelmä, ddPCR, toimii metsälieronäytteideni kohdalla. ddPCR-menetelmän toistettavuus tutkittiin vertaamalla aineistoa, jonka tuotti sama henkilö kahdesti ja toisaalta toinen henkilö. Vertasin myös saadaanko samat tulokset geenien ilmentymisen populaatiovertailussa sekä qPCR- että ddPCR-menetelmällä. Tutkin myös saadaanko samat tulokset, kun näytteistä on eristetty RNA kahdella eri menetelmällä. Toistettavuus osoittautui korkeaksi, mutta tulokset populaatiovertailusta eivät olleet samanlaiset kahdella eri menetelmällä. Erojen taustalla oli geenien ilmentymisen mittausten normalisointiin käytetty menetelmä, joten käyttämällä normalisoimismenetelmää, joka sopii sekä qPCR:ään että ddPCR:ään voi olla mahdollista saada yhteneviä tuloksia. Myöskään eri RNA:n eristysmenetelmillä ei saatu samoja tuloksia kaikissa tutkituista neljästä geenistä, eli RNA:n eristysmenetelmä saattaa vaikuttaa tuloksiin. ddPCR vaikuttaa hyvältä menetelmältä geenien ilmentymisen mittaamiseen, kunhan käytetään sopivaa normalisoimismenetelmää ja RNA:n laatu tarkistetaan.

Jatkossa metallikontaminaation vaikutuksia olisi hyvä tutkia myös muilla metalleilla kuin kuparilla ja sinkillä, ja vasteita myös muilla geeneillä kuin metallotioniinin geenillä, jotta selviäisi eri metallien vaikutukset myös muuhun kuin metallinsietokykyyn. Geenien ilmentymisen vaihtelua voisi tutkia myös laajemmin, sillä tutkimuksessani selvisi, että vaihtelu on suurta. Täten olisi hyvä tutkia sen vaikutukset esimerkiksi ekotoksikologisten testien tuloksiin, joiden pohjalta tehdään usein ympäristöä koskevia päätöksiä. Geneettisen diversiteetin vaikutusta voisi tutkia myös yksilöillä, joilla ei ole metallialtistushistoriaa sekä millainen geneettisen diversiteetin vaikutus on eri metalleilla saastutetuissa oloissa. On myös tärkeää tutkia millainen normalisoimismenetelmä sopii parhaiten ddPCR-menetelmälle, jotta sillä saadaan luotettavaa tietoa geenien ilmentymisestä.

REFERENCES

Adejuyigbe, C.O., Tian, G. & Adeoye, G. O. 2006. Microcosmic study of soil microarthropod and earthworm interaction in litter decomposition and nutrient turnover. *Nutr. Cycl. in Agroecosyst.* 75: 47-55.

Aguirre, J. D. & Marshall D. J. 2012. Genetic diversity increases population productivity in a sessile marine invertebrate. *Ecology* 93: 1134-1142.

Annila, A. & Annila, E. 2012. The significance of sex. *BioSystems* 110: 156-161.

Asensio, V., Rodriguez-Ruiz, A., Garmendia, L., Andre, J., Kille, P., Morgan, A.J., Soto, M. & Marigomez, I. 2013. Towards an intergrative soil health assessment strategy: A three tier (integrative biomarker response) approach with Eisenia fetida applied to soils subjected to chronic metal pollution. *Sci. Total Environ.* 442: 344-365.

Avolion, M. L., Chan, C. C., Weis, J. J. & Smith, M. D. 2014. The effect of genotype richness and genomic dissimilarity of *Andropogon gerardii* on invasion resistance and productivity. *Plant Ecol. Divers.* 8: 61-71.

Aziz, N.A., Morgan, A.J. & Kille, P. 1999. Metal resistance in earthworms; genetic adaptation or physiological acclimation. *Pedobiologia* 43: 594-601.

Bardgett, R. D. & van der Putten, W. H. 2014. Belowground biodiversity and ecosystem functioning. *Nature* 515: 505-511.

Barraclough, T. G. 2015. How do species interactions affect evolutionary dynamics across whole communities? *Annu. Rev. Ecol. Syst.* 46: 25-48.

Bassar, R. D., Marshall, M. C., Lopez-Sepulcre, A., Zandona, E., Auer, S. K., Travis, J., Pringle, C. M., Flecker, A. S., Thomas, S. A., Fraser, D. F. & Reznick, D. N. 2010. Local adaptation in Trinidadian guppies alters ecosystem processes. *PNAS* 107: 3616-3621.

Bengtsson, G., Nordstrom, S. & Rundgren, S. 1983. Population-density and tissue metal concentration of lumbricids in forest soils near a brass mill. *Environ. Pollut. Ser. A Ecol. Biol.* 30: 87–108.

Bengtsson, G., Ek, H. & Rundgren, S. 1992. Evolutionary response of earthworms to long-term metal exposure. *Oikos* 63:289–297.

Bindesbøl, A.M., Bayley, M., Damgaard, C., Hedlund, K. & Holmstrup, M. 2009. Changes in membrane phospholipids as a mechanistic explanation for decreased freeze tolerance in earthworms exposed to sublethal copper concentrations. *Environ. Sci. Technol* . 43: 5495–5500.

Blake, W., Balazsi, G., Kohanski, M., Isaacs, F., Murphy, K., Kuang, Y., Cantor, C., Walt & D., Collins, J. 2006. Phenotypic Consequences of Promoter-Mediated Transcriptional Noise. *Mol. Cell.* 24: 853-865.

Bradl, H. 2005. In: Hubbard, A. (Ed.), *Heavy Metals in the Environment: Origin, Interaction and Remediation*. Interface Science and Technology. Elsevier, Academic Press, Neubrucke, Germany.

Brulle, F., Mitta, G., Cocquerelle, C., Vieau, D., Lemiere, S., Lepretre, A. & Vandenbulcke, F. 2006. Cloning and real-time PCR testing of 14 potential biomarkers in *Eisenia fetida* following cadmium exposure. *Environ. Sci. Technol.* 40: 2844–2850.

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? *Comp. Biochem. Physiol. C* 144: 334-341.

Brulle, F., Lemiere, S., Waterlot, C., Doyay, F. & Vandenbulcke, F. 2011. Gene expression analysis of 4 biomarker candidates in *Eisenia fetida* exposed to an environmental metallic trace element gradient: A microcosm study. *Sci. Total Environ.* 409: 5470-5482.

Bustin, S.A. 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J. Mol. Endocrinol.* 25: 169-93.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vansompele, J. & Wittwer, C.T. 2009. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clin. Chem.* 55: 4.

Butlin, R. 2002. The costs and benefits of sex: new insights from old asexual lineages. *Nat. Rev. Gen.* 3: 311-317.

Butlin, R., Schön, I. & Martens, K. 1998. Asexual reproduction in nonmarine ostracods. *Heredity* 81: 473-480.

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

Calisi, A., Zaccarelli, N., Lionetto, M.G. & Schettino, T, 2013. Intergrated biomarker analysis in the earthworm *Lumbricus terrestris*: Application to the monitoring of soil heavy metal pollution. *Chemosphere* 90: 2637-2644.

Cortet, J., Gomot-De-Vauflery, A., Gomot, L., Poinsot-Balaguer, N., Texier, C. & Cluzeau, D. 1999. The use of invertebrate soil fauna in monitoring pollutant effects. *Eur. J. Soil Biol.* 35: 115-134.

Costa, D., Marien, J., Janssens, T. K. S., van Gestel, C. A. M., Driessen, G., Sousa, J. P., van Straalen, N. M. & Roelofs, D. 2012. Influence of adaptive evolution of cadmium tolerance on neutral and functional genetic variation in *Orchesella cincta*. *Ecotoxicol.* 21: 2078-2087.

Costa, D., Bouchon, D., van Straalen, N. M., Sousa, J. P. & Ribeiro, R. 2013. Copper tolerance and genetic diversity of *Porcellionides sexfasciatus* (isopoda) in a highly contaminated mine habitat. *Environ. Toxicol .Chem.* 32: 884-888.

Cragg, R. G. & Bardgett, R. D. 2001. How changes in soil faunal diversity and composition within a trophic group influence decomposition processes. *Soil Biol. Biochem.* 33: 2073-2081.

Dallinger, R. 1996. Metallothionein research in terrestrial invertebrates: synopsis and perspectives. *Comp. Biochem. Physiol. C* 113:125–133.

Dalziel, A., Rogers, S. & Schulte, P. 2009. Linking genotypes to phenotypes and fitness: how mechanistic biology can inform molecular ecology. *Mol. Ecol.* 18: 4997–5017.

Demuynck, S., Grumiaux, F., Mottier, V., Schikorski, D., Lemiere, S. & Lepretre, A., 2006. Metallothionein response following cadmium exposure in the oligochaete *Eisenia fetida*. *Comp. Biochem. Physiol. C* 144: 34–46.

Demuynck, S., Grumiaux, F., Mottier, V., Schikorski, D., Lemiere, S. & Lepretre, A. 2007. Cd/Zn exposure interactions on metallothionein response in *Eisenia fetida* (Annelida, Oligochaeta). *Comp. Biochem. Physiol. C* 145: 658-668.

Díaz Cosín, J., Novo, M. & Fernández, R. 2010. Reproduction of earthworms: sexual selection and parthenogenesis. In: Karaka A (ed) Biology of earthworms. Springer, Berlin, pp 69–86.

Dudycha, J., Brandon, C. & Deitz, K. 2012. Population genomics of resource exploitation: insights from gene expression profiles of two *Daphnia* ecotypes fed alternate resources. *Ecol. Evol.* 2: 329-340.

Duncan, C., Thompson, J.R. & Pettorelli, N. 2015. The quest for a mechanistic understanding of biodiversity – ecosystem services relationships. *Proc. R. Soc. B* 282: 20151348.

Edwards, C.A. & Bohlen, P.J. 1996. *Biology and Ecology of Earthworms*, third ed. Chapman & Hall, London.

Eisenhauer, N. & Schädler, M. 2011. Inconsistent impacts of decomposer diversity on the stability of aboveground and belowground ecosystem functions. *Oecologia* 165: 403-415.

Eisenhauer, N., Reich, P. B. & Isbell, F. 2012. Decomposer diversity and identity influence plant diversity effects on ecosystem functioning. *Ecology* 93: 2227-2240.

Ekschmitt, K., Klein, A., Pieper, B. & Wolters, V. 2001. Biodiversity and functioning of ecological communities - why is diversity important in some cases and unimportant in others? *J. Plant. Nutr. Soil Sci.* 164: 239-246.

Emerson, J.J. & Li, W. 2010. The genetic basis of evolutionary change in gene expression levels. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 365: 2581-2590.

Evans, E. W. 2016. Biodiversity, ecosystem functioning, and classical biological control. *Appl. Entomol. Zool.* 51: 173-184.

Fariña, J. M., Castilla, J. C. & Ojeda, F. P. 2003. The "idiosyncratic" effect of a "sentinel" species on contaminated rocky intertidal communities. *Ecol. Appl.* 13: 1533-1552.

Fernandes, I., Pascoal, C. & Cassio, F. 2011. Intraspecific traits change biodiversity effects on ecosystem functioning under metal stress. *Oecologia* 166: 1019-1028.

Fisker, K.V., Sørensen, J.G., Damgaard, C., Pedersen, K.L. & Holmstrup, M. 2011a. Genetic adaptation of earthworms to copper pollution: is adaptation associated with fitness costs in *Dendrobaena octaedra*? *Ecotoxicology* 20:563–573.

Fisker, K.V., Sørensen, J.G. & Holmstrup, M. 2011b. No costs on freeze tolerance in genetically copper adapted earthworm populations (*Dendrobaena octaedra*). *Comp. Biochem. Physiol.* C 154: 204-207.

Fisker, K.V., Holmstrup, M. & Sørensen, J.G. 2013. Variation in metallothionein gene expression is associated with adaptation to copper in the earthworm *Dendrobaena octaedra*. *Comp. Biochem. Physiol. C* 157: 220-226.

Galay Burgos, M., Winters, C., Sturzenbaum, S., Randerson, P., Kille, P. & Morgan, J. 2005. Cu and Cd effects on the earthworm *Lumbricus rubellu*s in the laboratory: Multivariate analysis of relationships between exposure, biomarkers, and ecologically relevant parameters. *Environ. Sci. Techol*. 39: 1757-1763.

Gessner, M.O., Swan, C. M., Dang, C. K., McKie, B. G., Bardgett, R. D., Wall, D. H. & Hättenschwiler, S. 2010. Diversity meets decomposition. *Trends Ecol. Evol.* 25: 372-380.

Giska, I., Babik, W., van Gestel, C. A., van Straalen, N. M. & Laskowski, R. 2015. Genome-wide genetic diversity of rove beetle populations along a metal pollution gradient. *Ecotoxicol. Environ. Saf.* 119: 98-105.

Grapputo, A. 2006. Development and characterization of microsatellite markers in the Colorado potato beetle, *Leptinotarsa decemlineata*. *Mol. Ecol. Notes* 6: 1177–1179.

Gravel, D., Bell, T., Barbera, C., Bouvier, T., Pommier, T., Venail, P. & Mouquet, N. 2011. Experimental niche evolution alters the strength of the diversity–productivity relationship. *Nature* 469: 89-92.

Gruber, C., Sturzenbaum, S., Gehrig, P., Sack, R., Hunziker, P., Berger, B. & Dallinger, R. 2000. Isolation and characterization of a self-sufficient one-domain protein — (Cd)-metallothionein from *Eisenia foetida*. *Eur. J. Biochem.* 267: 573–582.

Haimi, J., Knott, K. E., Selonen, S. & Laurikainen, M. 2006. Has long-term metal exposure induced changes in life history traits and genetic diversity of the enchytraeid worm *Cognettia sphagnetorum* (Vejd)? *Environ. Pollut.* 140: 463-470.

Haimi, J., Knott, K.E. & Yliniemi, I. 2007. Does metal contamination affect clonal diversity of the parthenogenetic earthworm *Dendrobaena octaedra*? *Eur. J. Soil Biol.* 43: 268-272.

Hansen, P. L., Holmstrup, M., Bayley, M., & Simonsen, V. 2006. Low genetic variation for *Dendrobaena octaedra* from Greenland compared to populations from Europe and North America: Refuge or selection? *Pedobiologia* 50: 225-234.

Harmon, L. J., Matthews, B., Des Roches, S., Chase, J. M., Shurin, J. B. & Schluter, D. 2009. Evolutionary diversification in stickleback affects ecosystem functioning. *Nature* 458: 1167-1170.

Harrison, P.A., Berry, P.M., Simpson, G., Haslett, J.R., Blicharska, M., Bucur, M., Dunford, R., Egoh, B., Garcia-Llorente, M., Geamănă, N., Geertsema, W., Lommelen, E., Meiresonne, L. & Turkelboom, F. 2014. Linkages between biodiversity attributes and ecosystem services: A systematic review. *Ecosystem services* 9: 191-203.

Heckmann, L., Sorensen, P., Krogh, P. & Sorensen, J. 2011. NORMA-Gene: A simple and robust method for qPCR normalization based on target gene data. *BMC Bioinformatics* 12:250.

Heemsbergen, D. A., Berg, M. P., Loreau, M., van Hal, J. R., Faber, J. H. & Verhoef , H. A. 2004. Biodiversity effects on soil processes explained by interspecific functional dissimilarity. *Science* 306: 1019-1020.

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F. & Vandesompele, J. 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Gen. Biol.* 8: R19.

Hindson, B.J., Ness, K.D., Masquelier, D.A., Belgrader, P., Heredia, N.J., Makarewicz, A.J., Bright, I.J., Lucero, M.Y., Hiddessen, A.L., Legler, T.C., Kitano, T.K., Hodel, M.R., Petersen, J.F., Wyatt, P.W., Steenblock, E.R., Shah, P.H., Bousse, L.J., Troup, C.B., Mellen, J.C., Wittmann, D.K., Erndt, N.G., Cauley, T.H., Koehler, R.T., So, A.P., Dube, S., Rose, K.A., Montesclaros, L., Wang, S., Stumbo, D.P., Hodges, S.P., Romine, S., Milanovich, F.P., White, H.E., Regan, J.F., Karlin-Neumann, G.A., Hindson, C.M., Saxonov, S. & Colston, B.W. 2011. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. *Anal. Chem.* 83: 8604–8610.

Holmstrup, M., Sørensen, J.G., Overgaard, J., Bayley, M., Bindesbøl, 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.

Hongell, K. & Terhivuo, J. 1989. Chromosomal status of the parthenogenetic earthworm *Dendrobaena octaedra* (Sav)(Oligochaete, Lumbricidae) in southern Finland. *Hereditas* 110: 179-182.

Hooper, D.U., Adair, E.C., Cardinale, B.J., Byrnes, J.E.K., Hungate, B.A., Matulich, K.L., Gonzales, A., Duffy, J.E., Gamfeldt L. & O'Connor, M.I. 2012. A global synthesis reveals biodiversity loss as a major driver of ecosystem change. *Nature* 486: 105-108.

Huggett, J., Dheda, K., Bustin, S. & Zumla, A. 2005. Real-time RT-PCR normalisation; strategies and considerations. *Genes Immun.* 6: 279–284.

Huggett, J.F., Foy, C.A., Benes, V., Emslie, K., Garson. J.A., Haynes, R., Hellemans, J., Kubista, M., Mueller, R.D., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T. & Bustin, S.A. 2013. The digital MIQE guidelines: Guidelines for Minimum Information for Publication of Quantitative Digital PCR Experiments. *Clin. Chem.* 59: 892–902.

Hughes, A. R. 2014. Genotypic diversity and trait variance interact to affect marsh plant performance. *J. of Ecol.* 102: 651-658.

Hughes, A. R., Inouye, B. D., Johnson, M. T. J., Underwood, N. & Vellend, M. 2008. Ecological consequences of genetic diversity. *Ecol. Letters* 11: 609-623.

Hättenschwiler, S., Tiurnov, A. V. & Scheu, S. 2005. Biodiversity and litter decomposition in terrestrial ecosystems. *Annu. Rev. Ecol. Syst.* 36: 191-218.

Janssens, T.K.S., Roelofs, D. & van Straalen, N.M. 2009. Molecular mechanisms of heavy metal tolerance and evolution in invertebrates. *Insect Sci.* 16: 3–18.

Johnson, D. W., Freiwald, J. & Bernardi, G. 2016. Genetic diversity affects the strength of population regulation in a marine fish. *Ecology* 97: 627-639.

Johnston, E. L. & Roberts, D. A. 2009. Contaminants reduce the richness and evenness of marine communities: A review and meta-analysis. *Environ. Pollut.* 157: 1745-1752.

Johnston, E. L., Mayer-Pinto, M. & Crowe, T. P. 2015. Chemical contaminant effects on marine ecosystem functioning. *J. of Appl. Ecol.* 52: 140-149.

Jung, J., Philoppot, L. & Park, W. 2016. Metagenomic and functional analyses of the consequences of reduction of bacterial diversity on soil functions and bioremediation in diesel-contaminated microcosms. *Scientific Reports* 6: 23012.

Keightley, P.D. & Caballero, A. 1997. Genomic mutation rates for lifetime reproductive output and lifespan in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* 94: 3823-3827.

Kille, P., Andre, J., Anderson, C., Ang, H., Bruford, M., Bundy, J., Donnelly, R., Hodson, M., Juma, G., Lahive, E., Morgan, A., Sturzenbaum, S. & Spurgeon, D. 2013. DNA sequence variation and methylation in an arsenic tolerant earthworm population. *Soil Biol. Biochem.* 57: 524-532.

Knott, K.E. & Haimi, J. 2010. High mitochondrial DNA sequence diversity in the parthenogenetic earthworm *Dendrobaena octaedra*. *Heredity* 105: 341–347.

Kozera, B. & Rapacz, M. 2013. Reference genes in real-time PCR. *J. Appl. Genetics* 54: 391-406.

Kozlov, M.V. & Zvereva, E.L. 2011. A second life for old data: global patterns in pollution ecology revealed from published observational studies. *Environ. Pollut.* 159: 1067-1075.

Laakso, J. & Setälä, H. 1999. Sensitivity of primary production to changes in the architecture of belowground food webs. *Oikos* 87: 57-64.

Langdon, C.J., Piearse, T.G., Meharg, A.A. & Semple, K.T. 2003. Inherited resistance to arsenate toxicity in two populations of *Lumbricus rubellus*. *Environ. Toxicol. Chem.* 22(10): 2344-2348.

Lavelle, P., Decaens, T., Aubert, M., Barot, S., Blouin, M., Bureau, F., Margerie, P., Mora, P. & Rossi, J.P. 2006. Soil invertebrates and ecosystem services. *Eur. J. Soil Biol.* 42: S3-S15.

Levine, M. & Tjian, R. 2003. Transcription regulation and animal diversity. *Nature* 424: 147-151.

Li, J., Duan, H., Li, S., Kuang, J., Zeng, Y. & Shu, W. 2010. Cadmium pollution triggers a positive biodiversity–productivity relationship: evidence from a laboratory microcosm experiment. *J. of Appl .Ecol.* 47: 890-898.

Lopez-Maury, L., Marquerat, S. & Bähler, J. 2008. Tuning gene expression to changing environments: from rapid responses to evolutionary adaptation. *Nature Reviews Genetics* 9: 583-593.

Lukkari, T. & Haimi, J. 2005. Avoidance of Cu- and Zn-contaminated soil by three ecologically different earthworm species. *Ecotox. Environ. Safe.* 62: 35-41.

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

Lukkari, T., Taavitsainen, M., Väisänen, A. & Haimi, J. 2004b. Effects of heavy metals on earthworms along contamination gradients in organic rich soils. *Ecotox. Environ. Saf.* 59: 340-348.

Lushai, G. & Loxdale, H.D. 2002. The biological improbability of a clone. *Genet. Res. Camb.* 79: 1-9.

Marino, F., Sturzenbaum, S.R., Kille, P. & Morgan, A.J. 1998. Cu–Cd interactions in earthworms maintained in laboratory microcosms: the examination of a putative copper paradox. *Comp. Biochem. Physiol. C* 120: 217–223.

Matthews, B., Narvani, A., Hauch, S., Nonaka, E., Peter, H., Yamamichi, M., Sullam, K. E., Bird, K. C., Thomas, M. K., Hanley, T. C. & Turner, C. B. 2011. Toward an integration of evolutionary biology and ecosystem science. *Ecol. Letters* 14: 690-701.

Meyer, S.T., Koch, C. & Weisser, W.W. 2015. Towards a standardized Rabid Ecosystem Function Assessment (REFA). *Trends Ecol. Evol.* 30: 390-7.

Nahmani, J., Hodson, M. E. & Black, S. 2007. A review of studies performed to assess metal uptake by earthworms. *Environ. Pollut.* 145: 402-424.

Neiman, M. & Schwander, T. 2011. Using parthenogenetic lineages to identify advantages of sex. *Evol. Biol.* 38: 115–123

Niemeyer, J.C., Nogueira, M.A., Carvalho, G.M., Cohin-De-Pinho, S.J., Outeiro, U.S., Rodrigues, G.G., da Silva, E.M. & Sousa, J.P. 2012. Functional and structural parameters to assess the ecological status of a metal contaminated area in the tropics. *Ecotoxicol. Environ. Saf.* 86: 188-97.

Nota, B., de Korte, M., Ylstra, B., van Straalen, N.M. & Roelofs, D. 2013. Genetic variation in parthenogenetic collembolans is associated with differences in fitness and cadmium-induced transcriptome. *Environ. Sci. Technol.* 47: 1155−1162.

Omodeo P. 1955. Cariologia dei Lumbricidae. II. Contributo. *Caryologia* 8: 135–178.

Ong-Abdullah,M.,Ordway,J.,Jiang,N.,Ooi,S,Kok,S.,Sarpan,N.,Azimi,N.,Hashim, A.,Ishak, Z., Rosli, S.,Malike,F.,Bakar,N., Marjuni, M., Abdullah, N.,Yaakub, Z.,Amiruddin,M.,Nookiah, R.,Singh, R., Low,E., Chan,K., Azizi,N., Smith, S.,Bacher,B.,Budiman,M.,Van Brunt,A.,Wischmeyer,C., Beil,M.,Hogan,M.,Lakey, N.,Lim, C.,Arulandoo,X., Wong,C.,Choo, C., Wong,W., Kwan,Y., Alwee,S., Sambanthamurthi,R. & Martienssen,R. 2015. Loss of *Karma* transposon methylation underlies the mantled somaclonal variant of oil palm. *Nature* 24: 525-533.

Pauwels, M., Frenot, H., Souleman, D. & Vandenbulcke, F. 2013. Using biomarkers in an evolutionary context: Lessons from the analysis of biologi-

cal responses of oligochaete annelids to metal exposure. *Environ. Pollut.* 179: 343-350.

Prieto, I., Violle, C., Barre, P., Durand, J., Ghesquiere, M. & Litrico, I. 2015. Complementary effects of species and genetic diversity on productivity and stability of sown grasslands. *Nature Plants* 1: 15033.

Pritchard, C., Coil, D., Hawley, S., Hsu, L. & Nelson, P. 2006. The contributions of normal variation and genetic background to mammalian gene expression. *Genome Biology* 7: R26.

Reusch, T. R. & Hughes, A. R. 2006. The Emerging Role of Genetic Diversity for Ecosystem Functioning: Estuarine Macrophytes as Models. *Estuar. Coast.* 29: 159-164.

Raj, A. & van Oudenaarden, A. 2008. Nature, nurture, or change: Stochastic Gene Expression and Its Consequences. *Cell* 135: 216-226.

Roelofs, D., Aarts, M.G.M., Schat, H. & van Straalen, N.M. 2008. Functional ecological genomics to demonstrate general and specific responses to abiotic stress. *Funct. Ecol.* 22: 8-18.

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

Romero, I.G., Ruvinsky, I. & Gilad, Y. 2012. Comparative studies of gene expression and the evolution of gene regulation. *Nat. Rev. Genet.* 13: 505-16.

Rosa, B., Jasmer, D. & Mitreva, M. 2014. Genome-Wide Tissue-Specific Gene Expression, Co-expression and Regulation of Co-expressed Genes in Adult Nematode *Ascaris suum*. *PLoS Negl. Trop. Dis.* 8: e2678.

Ross, K., Cooper, N., Bidwell, J.R & Elder, J. 2002. Genetic diversity and metal tolerance of two marine species: a comparison between populations from contaminated and reference sites. *Mar. Pol. Bull.* 44: 671-679.

Rozen, A. 2003. Do earthworms (*Dendrobaena octaedra*) from differently polluted forests vary in life history parameters? *Eur. J. Soil Biol.* 39: 73–77.

Rozen, A. 2006. Effect of cadmium on life-history parameters in *Dendrobaena octaedra* (Lumbricidae: Oligochaeta) populations originating from forests differently polluted with heavy metals. *Soil Biol. Biochem.* 38: 489-503.

Rue, P. & Arias, A.M. 2015. Cell dynamics and gene expression control in tissue homeostasis and development. *Mol. Syst. Biol.* 11: 792.

Rundgren, S. 1975. Vertical Distribution of Lumbricids in Southern Sweden. *Oikos* 26: 299-306.

Salemaa, M. 2003. Responses of the understory vegetation of boreal forests to heavy metal loading. Dissertation, University of Helsinki.

Salminen, J. & Haimi, J. 1999. Horizontal distribution of copper, nickel and enchytraeid worms in polluted soil. *Environ. Pollut.* 104: 351-358.

Scheu, S., Schlitt, N., Tiunov, A. V., Newington, J. E. & Jones, T. H. 2002. Effects of the presence and community composition of earthworms on microbial community functioning. *Oecologia* 133: 254-260.

Schöb, C., Kerle, S., Karley, A. J., Morcillo, L., Pakeman, R. J., Newton, A. C. & Brooker, R. W. 2014. Intraspecific genetic diversity and composition modify species-level diversity-productivity relationships. *New Phytol.* 205: 720-730.

Signorovitch, A., Hur, J., Gladyshev, E. & Meselson, M. 2015. Allele Sharing and Evidence for Sexuality in a Mitochondrial Clade of Bdelloid Rotifers. *Genetics* 200: 581-90.

Simonsen, V. & Holmstrup, M. 2008. Deviation from apomictic reproduction in *Dendrobaena octaedra*? *Hereditas* 145: 212-214.

Simonsen, V., Laskowski, R., Bayley, M. & Holmstrup, M. 2008. Low impact of metal pollution on genetic variation in the earthworm *Dendrobaena octaedra* measured by allozymes. *Pedobiologia* 52: 51-60.

Spurgeon, D.J. & Hopkin, S.P. 1999. Tolerance to zinc in populations of the earthworm *Lumbricus rubellus* from uncontaminated and metal-contaminated ecosystems. *Arch. Environ. Contam. Toxicol.* 37: 332-337.

Spurgeon, D.J., Weeks, J.M. & van Gestel, C.A.M. 2003. A summary of eleven years progress in earthworm ecotoxicology. *Pedobiologia* 47: 588-606.

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. *Comp. Biochem. Physiol. C* 138: 11-21.

Spurgeon, D.J., Ricketts, H., Svendsen, C., Morgan, A.J. & Kille, P. 2005a. Hierarchical responses of soil invertebrates (earthworms) to toxic metal stress. *Environ. Sci. Technol.* 39: 5327-5334.

Spurgeon, D.J., Svendsen, C., Lister, L.J., Hankard, P. K. & Kille, P. 2005b. Earthworm responses to Cd and Cu under fluctuating environmental conditions: A comparison with results from laboratory exposures. *Environ. Pollut.* 136: 443-452.

Sturzenbaum, S.R., Kille, P. & Morgan, A.J. 1998. The identification, cloning and characterization of earthworm metallothionein. *FEBS Letters* 431: 437-442.

Sturzenbaum, S.R., Georgiev, O., Morgan, A.J. & Kille, P. 2004. Cadmium detoxification in earthworms: From genes to cells. *Environ. Sci. Technol.* 38: 6283-6289.

Sturzenbaum, S.R., Andre, J., Kille, P. & Morgan, A.J. 2009. Earthworm genomes, genes and proteins: the (re)discovery of Darwin's worms. *Proc. R. Soc. B* 276: 789-797.

Swain, S.C., Keusekotten, K., Baumeister, R. & Sturzenbaum, S.R. 2004. *C. elegans* metallothioneins: New insights into the phenotypic effects of cadmium toxicosis. *J. Mol. Biol.* 341: 951-959.

Sylvain, Z. A. & Wall, D. H. 2011 Linking soil biodiversity and vegetation: Implications for changing planet. *Am. J. of Botany* 98: 517-527.

Terhivuo, J. 1988. The Finnish Lumbricidae (Oligichaeta) fauna and its formation. *Ann. Zool. Fennici.* 25: 229-247.

Terhivuo, J. & Saura, A. 1990. Allozyme variation in parthenogenetic *Dendrobaena octaedra* (Oligochaeta, Lumbricidae) populations of eastern Fennoscandia. *Pedobiologia* 34: 113-139.

Terhivuo, J. & Saura, A. 2006. Dispersal and clonal diversity of North-European parthenogenetic earthworms. *Biol. Invasions* 8: 1205-1218.

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.

Tosza, E., Dumnicka, E., Niklinska, M. & Rozen, A. 2010. Enchytraeid and earthworm communities along a pollution gradient near Olkusz (southern Poland). *Eur. J. Soil Biol.* 46: 218–224.

Ungherese, G., Mengoni, A., Somigli, S., Baroni, D., Focardi, S. & Ugolini, A. 2010. Relationship between heavy metals pollution and genetic diversity in Mediterranean populations of the sandhopper *Talitrus saltator* (Montagu) (Crustacea, Amphipoda). *Environ. Pollut.* 158: 1638-1643.

Valko, M., Morris, H. & Cronin, M.T.D. 2005. Metals, toxicity and oxidative stress. *Curr. Med. Chem.* 12: 1161–1208.

van Straalen, N.M. 2002. Assessment of soil contamination - a functional perspective. *Biodegradation* 13: 41-52.

van Straalen, N. M. & Timmermans, M. J. T. N. 2007. Genetic variation in toxicant-stressed populations: An evaluation of the "genetic erosion" hypothesis. *Hum. and Ecol. Risk Asses.* 8: 983-1002.

Velavan, T.P., Schulenburg, H. & Michiels, N.K. 2007. Development and characterization of novel microsatellite markers for the common earthworm (*Lumbricus terrestris* L.). *Mol. Ecol .Notes* 7: 1060-1062.

Verhoef, H.A. 1996. The role of soil microcosms in the study of ecosystem processes. *Ecol.* 77: 685-690.

Viney, M. & Reece, S. 2013. Adaptive noise. *Proc. R. Soc. B* 280: 20131104.

Wagg, C., Bender, S. F., Widmer, F. & van der Heijden, M. G. A. 2014. Soil biodiversity and soil community composition determine ecosystem multifunctionality. *Proc. Natl. Acad. Sci. USA* 111: 5266-5270.

Weeks, J.M., Spurgeon, D.J., Svedsen, C., Hankard, P.K., Kammenga, J.E., Dallinger, R., Köhler, H., Simonsen, V. & Scott-Fordsmand, J. 2004. Critical analysis of soil invertebrate biomarkers: A field case study in Avonmouth, UK. *Ecotoxicology* 13:.817-822.

Whitlock, R. 2104. Relationships between adaptive and neutral genetic diversity and ecological structure and functioning: a meta-analysis. *J. of Ecol.* 102: 857-872.

Wolff, G. 1996. Variability in Gene Expression and Tumor Formation within Genetically Homogeneous Animal Populations in Bioassays. *Fundam. Appl. Toxicol.* 29: 176-184.

Wong, A., Gottesman, I. & Petronis, A. 2005. Phenotypic differences in genetically identical organisms: the epigenetic perspective. *Hum. Mol. Genet.* 14: R11-R18.

Wymore, A. S., Keeley, A. T. H., Yturralde, K. M., Schroer, M. L., Propper, C. R. & Whitman, T. G. 2011. Genes to ecosystems: exploring the frontiers of ecology with one of the smallest biological units. *New Phytol.* 191: 19-36.

Zane, L., Bargelloni, L. & Patarnello, T. 2002. Strategies for microsatellite isolation: a review. *Mol. Ecol.* 11: 1-16.

Zimmer, M., Kautz, G. & Topp, W. 2005. Do woodlice and earthworms interact synergistically in leaf litter decomposition? *Funct. Ecol.* 19: 7-16.

ORIGINAL PAPERS

I

METALLOTHIONEIN GENE EXPRESSION DIFFERS IN EARTHWORM POPULATIONS WITH DIFFERENT EXPOSURE HISTORY

by

Marina Mustonen, Jari Haimi, Ari Väisänen & K. Emily Knott 2012

Ecotoxicology 23: 1732-1743

Ecotoxicology (2014) 23:1732–1743
DOI 10.1007/s10646-014-1338-z

Metallothionein gene expression differs in earthworm populations with different exposure history

M. Mustonen · J. Haimi · A. Väisänen · K. E. Knott

Accepted: 18 August 2014 / Published online: 2 September 2014

Abstract Metals are persistent pollutants in soils that can harm soil organisms and decrease species diversity. Animals can cope with metal contamination with the help of metallothioneins, small metal-binding proteins involved in homeostasis and detoxification of metals. We studied the expression of metallothionein with qPCR in a small, epigeic earthworm, *Dendrobaena octaedra.* We compared expression patterns and metal body content in earthworms collected from two sites with different metal contamination histories: Harjavalta, contaminated by a Cu–Ni smelter operational for over 50 years, and Jyväskylä, an uncontaminated site. Earthworms from both sites were also experimentally exposed to different concentrations of Cu (control, 50, 100 or 200 mg/kg) or Zn (control, 75, 150 or 300 mg/kg) for 7, 14 or 28 days to determine if there is a time related dose–response in gene expression. Population comparison showed that metallothionein expression was higher in earthworms from the contaminated site. In the exposure experiment, exposure time affected expression, but only in the earthworms from the uncontaminated site, suggesting that there is a delay in the metallothionein response of earthworms in this population. In contrast, earthworms from the contaminated site showed higher and constant levels of metallothionein expression at all exposure concentrations and durations. The constant metallothionein expression in earthworms from the contaminated site suggests that inducibility of metallothionein response could be lost in earthworms with metal exposure history. Adaptation of *D. octaedra* to metal exposure could explain the differences between the populations and explain the persistence of this species in contaminated forest soils.

Keywords *Dendrobaena octaedra* · Adaptation · Biomarker · Metal contamination

Electronic supplementary material The online version of this article (doi:10.1007/s10646-014-1338-z) contains supplementary material, which is available to authorized users.

M. Mustonen (✉) · J. Haimi · K. E. Knott
Department of Biological and Environmental Science, University of Jyvaskyla, P.O. Box 35, FI-40014 Jyvaskyla, Finland
e-mail: marina.s.mustonen@jyu.fi

A. Väisänen
Department of Chemistry, University of Jyvaskyla, P.O. Box 35, FI-40014 Jyvaskyla, Finland

Introduction

Human activities, such as agriculture, mining, energy production, transportation, and waste disposal, are major sources of metal contamination in the environment (Bradl 2005). Some metals are essential for organisms (e.g. Cu and Zn), since they are needed for metabolic and signaling pathways, but these metals can become toxic at high concentrations. On the other hand, non-essential metals (e.g. Cd and Hg) can be toxic even at low concentrations (Valko et al. 2005; Roelofs et al. 2008). Exposure to both essential and non-essential metals can lead to the production of reactive radicals that induce e.g. genotoxic damage (Valko et al. 2005). Organisms that cannot cope with metal toxicity cannot persist in metal contaminated environments. Consequently, metal contamination can decrease species diversity (Kozlov and Zvereva 2011) which can have major, and often detrimental, effects on ecosystem functioning (Hooper et al. 2012).

In terrestrial ecosystems, metals accumulate in the litter layer and soil and they are only slowly eliminated, so many soil organisms are faced with the challenges of metal

exposure and toxicity (Bengtsson et al. 1983; Roelofs et al. 2008; Simonsen et al. 2008). Understanding the effects of metal exposure on earthworms is particularly important, since earthworms are efficient decomposers and contribute considerably to nutrient mineralization and soil structure (Edwards and Bohlen 1996; Spurgeon et al. 2003; Lavelle et al. 2006; Sturzenbaum et al. 2009). Earthworms are often used as indicator organisms to monitor the health of ecosystems (Lavelle et al. 2006; Nahmani et al. 2007). However, the response of earthworms to metal contamination in terms of loss or persistence of species may only be evident after significant contamination of the environment (Spurgeon et al. 2005a).

In order to advance detection of the detrimental effects of metal contamination, molecular genetic approaches have been used in ecotoxicological research (Roelofs et al. 2008; Sturzenbaum et al. 2009). For example, organismal response to metal contamination can be measured on the genetic level as the expression of stress-proteins (e.g. heat shock proteins or HSPs) and other proteins that have a function in homeostasis and the detoxification of metals, e.g. metallothioneins (Cortet et al. 1999; Weeks et al. 2004). Metallothioneins are small (6–8 kDa), hydrophilic metal-binding proteins, which are found in many organisms, including prokaryotes, invertebrates, fish, mammals and plants (Dallinger 1996; Janssens et al. 2009). Metallothioneins are important for metal management and tolerance since they can detoxify non-essential metals and regulate the metabolism of essential metals (Cortet et al. 1999; Valko et al. 2005). Metallothioneins in earthworms were first identified by Sturzenbaum et al. (1998) and now they have been studied in many earthworm species, including *Lumbricus rubellus* (Marino et al. 1998; Galay Burgos et al. 2005; Spurgeon et al. 2005a, 2005b; Bundy et al. 2008), *Lumbricus terrestris* (Calisi et al. 2013), *Eisenia fetida* (Gruber et al. 2000; Brulle et al. 2006, 2007, 2011; Demuynck et al. 2006, 2007; Asensio et al. 2013), *Aporrectodea tuberculata* (Lukkari et al. 2004a) and *D. octaedra* (Fisker et al. 2013). Since several studies have found clear time- and dose–response relationships between metal exposure and metallothionein expression or concentration (e.g. Gruber et al. 2000; Brulle et al. 2006; Demuynck et al. 2007), metallothionein is considered to be a good biomarker for the sublethal effects of metal contamination.

Our study species, the epigeic earthworm *D. octaedra,* can be the dominant earthworm species in northern coniferous forests and tundra (Edwards and Bohlen 1996; Terhivuo and Saura 2006). *D. octaedra* is often exposed to harmful anthropogenic substances, especially metals, which have accumulated in boreal forest ecosystems (Bengtsson et al. 1983). Although sensitive to metals (Bindesbøl et al. 2009), *D. octaedra* can persist in contaminated soils with high concentrations of metals (Bengtsson et al. 1983, 1992; Rozen 2003, 2006; Lukkari et al. 2004b; Simonsen et al. 2008; Tosza et al. 2010; Holmstrup et al. 2011). *D. octaedra* avoids metals even at low concentrations (Lukkari and Haimi 2005), which probably contributes to its survival in contaminated habitats. In addition, *D. octaedra* can adapt to metal exposure. Fisker and colleagues (2011a) found that growth and reproduction was higher and maturation time was shorter in F1-generation *D. octaedra* that had Cu exposure history than in earthworms without exposure history, both in control and Cu-spiked soils. In addition, Fisker and colleagues (2013) showed that F2-generation earthworms, which were raised in uncontaminated soil, but originating from populations with metal exposure history, expressed metallothionein more quickly and in greater quantities when exposed to metal contaminated soil than did reference individuals.

Given the ability of *D. octaedra* to adapt to metal exposure (Fisker et al. 2011a, 2011b, 2013), we expect that earthworms collected from populations with different metal exposure histories will differ in metallothionein gene expression. In other species, adaptation to metal exposure has led to over-expression of genes associated with the stress response (Roelofs et al. 2008, 2009). We examined individual variation in metallothionein expression in *D. octaedra* earthworms collected from populations with different metal exposure histories. We also tested whether there would be a time- or dose–response in metallothionein expression among individuals originating from the different populations when exposed to three concentrations of Cu or Zn for 7, 14 or 28 days. Cu and Zn were chosen for the exposure experiment because they are the most abundant contaminants in our contaminated study site and also because they are both essential metals. In addition, we compared metallothionein expression to metal body content of the earthworms in both our population comparison and the exposure experiment. Since metal tolerance can be accompanied by costs, e.g. reduced tolerance to other stress factors (Posthuma and van Straalen 1993), it is important to clarify possible different responses of populations with or without history to metal exposure.

Materials and methods

Sampling of earthworms

Dendrobaena octaedra were collected from two locations: a metal contaminated site in Harjavalta (South-West Finland, 61°18′50″N, 22°08′30″E) and an uncontaminated site in Jyväskylä (Central Finland, 62°12′39″N, 25°44′13″E). The main emission source in Harjavalta is a Cu-Ni smelter. The Cu smelter has been operational since 1945 and the Ni smelter since 1960. Emissions from the smelters over the

Table 1 Mean metal concentrations, pH and soil organic matter (SOM) of soil from the contaminated (Harjavalta) and uncontaminated (Jyväskylä) study sites

	Cu	Zn	Cd	Ni	Pb	As	Cr	pH	SOM
Contaminated	823.5	473.6	<LOQ	262.7	32.72	6.22	5.334	5.2	72.21
Uncontaminated	9.792	27.35	<LOQ	4.237	22.55	<LOQ	<LOQ	4.3	68.60

Some metal concentrations were under the limit of quantification (<LOQ)

decades have led to the accumulation of metals in the soil, (Table 1) despite the fact that emissions were significantly reduced in the early 1990s after the installation of new filters (Salemaa 2003). There is no history of metal emissions in Jyväskylä (Table 1). The contaminated site is a birch dominated forest on sandy soil, and the uncontaminated site is a spruce dominated forest on moraine soil. Soil samples were taken from both sites (three 5.5 cm diameter core samples of organic soil from five locations at the sampling site) to determine metal concentration, pH and organic matter content of the soil (Table 1).

Earthworms were collected from both sites at two times: August 2011 and September or October 2012. Both times, 24 individuals were collected from each site for comparison of metallothionein expression. Individuals were collected by hand, stored in a bucket with soil and humus from the sampling site and transferred to the laboratory where they were kept at +15 °C. Before RNA extraction, the earthworms were moved to jars containing moist paper towel and kept at +15 °C for 2 days to allow the earthworms to empty their guts. The paper towel was changed after 1 day to prevent coprophagy. The anterior end (ca. 5 mm) of each earthworm was preserved for RNA extraction. The tissue was removed using scissors or a scalpel, placed in a 1.5 ml microcentrifuge tube and immediately frozen in liquid nitrogen. The rest of the body was reserved for metal content analysis. It was placed in a separate 1.5 ml microcentrifuge tube and also frozen with liquid nitrogen. Both samples were stored at −80 °C until the RNA extraction and metal content analysis could be completed (see below). RNA extraction was performed within a few days of sampling; however, metal content analysis was performed later (November 2012) when all the samples could be analyzed simultaneously.

Additional samples (110–130 individuals) were collected from each site in September and November 2012 for use in the exposure experiment, which was conducted in April 2013 (see below). All individuals sampled for use in the exposure experiment were kept at +15 °C in uncontaminated soil prior to the experiment.

Exposure experiment

Soil used in the exposure experiment was collected from an uncontaminated mature spruce forest in Jyväskylä (soil water content = 75.0 %; pH 4.6; organic matter content = 82.5 %), and it was novel soil for earthworms from both populations. Three different concentrations of Cu or Zn (50, 100, 300 Cu mg/kg or 75, 150, 300 Zn mg/kg) and an uncontaminated control were used (following Lukkari and Haimi 2005). The appropriate amounts of copper chloride ($CuCl_2 \cdot H_2O$) or zinc chloride ($ZnCl_2$) were added to 200 ml of water and thoroughly mixed with 0.5 kg soil (fresh mass). Pure water was added to the control soil. After addition of the metals, pH of the soil decreased, ranging from 4.3 to 4.5, and soil water content increased to 82.2 %. The spiked soil was incubated for 7 days at +15 °C before the experiment.

Each experiment (with Cu or Zn contamination) consisted of four treatments: low, medium and high metal concentrations and an uncontaminated control. Glass jars (Ø 5.5 cm) with perforated lids were used for the treatments and there were five replicate jars per treatment and population. Each jar contained 50 g of soil and three earthworms (adult or sub-adult). Earthworms were rinsed in water and weighed before the experiment. Throughout the experiment, the jars were incubated at +15 °C and water was added each week to replace what was lost from evaporation. Earthworms were weighed weekly and biomass did not change (Kruskall-Wallis test, $\chi^2 < 5.17$, $df = 3$, $p > 0.1$). One earthworm from each jar was removed after 7 days of exposure, a second after 14 days and the third at the end of the experiment, after 28 days of exposure. Part of the sampled earthworms was preserved for analysis of metallothionein expression and the remainder was preserved for metal content analysis (as described above). Samples of the earthworms from the exposure experiment were stored at −80 °C for up to 30 days prior to RNA extraction and metal content analysis (see below). There was some mortality (30 deaths) during the experiment. Thus, the final number of individuals analyzed was 3–5 per treatment at each sampling point (180 samples altogether).

RNA extraction and qPCR

RNA was extracted using the Aurum Total RNA mini kit (Bio-Rad) following the manufacturer's protocol, with DNase treatment. RNA concentration was determined with the Qubit RNA Assay Kit and Qubit Fluorometer (Invitrogen, Thermo Fisher Scientific). A concentration of 35 μg/mL of

Table 2 Primer sequences used in qPCR and reaction efficiencies

Gene	Primer (5′-3′)	Efficiency %
Metallothionein, mt-2	F: ACA CTC AGT GCT GTG GCA GCG	103.5
	R: GGC TGC GCA CTT GCA GGC	
18S-DNA	F: ACC ACA TCC AAG GAA GGC AG	93.3
	R: CCC GAG ATC CAA CTA CGA GC	
28S-DNA	F: TGG TGG AGG TCC GCA GCG AT	90.5
	R: CGT TTC GTC CCC AAG GCC TC	

RNA was used for cDNA synthesis, which was done using the iScript cDNA synthesis kit (Bio-Rad) following the manufacturer's protocol. cDNA was then diluted 1:5.

Real-time quantitative polymerase chain reactions (qPCR) were done with IQ SYBR green super mix(Bio-Rad) using species specific primers to the metallothionein gene (mt-2) and two reference genes (18S-rDNA and 28S-rDNA). Prior to our analysis, qPCR conditions were optimized and efficiencies were checked with a dilution series (5 points, 10-fold dilutions) (Table 2). In each reaction we used 1 µl of cDNA template (approximately 0.7 ng) and 0.5 µM of each primer in a final reaction volume of 20 µl (concentrations other of reagents in the IQ SYBR green super mix are pre-optimized by the manufacturer). Three replicate reactions for each sample were performed. An inter-run calibrator was prepared by combining equal quantities of cDNA from all samples, and five replicate reactions of this calibrator were included in each qPCR run. We used a CFX96 C1000 Touch Thermal Cycler (Bio-Rad) with the following protocol: 94 °C for 2 min and then 40 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 15 s followed by a plate read. Finally, melting curve analysis was done by heating the product at 95 °C for 10 s and then increasing the temperature from 55 °C to 95 °C in 0.5 °C increments followed by a plate read.

Some samples used in the population comparison failed to amplify and were deleted from the dataset. These failures were likely due to incorrect species identification of the sample. As a result, there were only 41 samples from the uncontaminated site for analysis. Consequently, we chose to analyze only 42 samples from the contaminated site in order to keep the datasets even.

Metal analyses

An ultrasound-assisted extraction method was used to measure metal content in our experimental soils and in the bodies of the earthworms from the population comparison and from the exposure experiment. The previously frozen earthworms were dried and weighed prior to measurement. For the experimental soils, three replicates of 500 mg dried soil samples were measured. Earthworm samples were digested with a mixture of 2 ml of aqua regia plus 2 ml of ultrapure water whereas the soil samples were digested with a mixture of 5 ml of aqua regia plus 5 ml of ultrapure water. The digestion was performed with an ultrasound water bath (+70 °C, 5 × 3 min for earthworms and 3 × 3 min for soil, shaking after each 3 min sonication). Solutions were filtered (Whatman No. 41 filter paper), and prior to analysis, earthworm samples were diluted to 10 ml with ultrapure water and transferred to 50 ml plastic bottles, whereas soil samples were diluted to 50 ml with ultrapure water and transferred to 100 ml plastic bottles. As, Cd, Pb, Cu, Ni, Cr and Zn content was measured in the earthworms for the population comparison and Cu and Zn content was measured in the earthworms from the exposure experiment since they were exposed only to Cu and Zn. All metal content measurements were performed with a Perkin Elmer Optima 8300 using inductively coupled plasma optical emission spectrometry.

Data analysis and statistics

Raw data from the qPCR runs were first analyzed with qBase + (Biogazelle) to adjust results for the different reaction efficiencies and to perform inter-run calibration. qBase+ was also used to check the stability of the reference gene expression and to calculate normalized relative quantities of the target gene. Expression of the reference genes was stable (data not shown). Two outlier values of very low metallothionein expression (Cq > 35) detected in earthworms from the exposure experiment were removed from the dataset. Raw data from the metal content analyses were checked for conformity to a normal distribution using a Kolmogorov–Smirnov test and for homogeneity of variances with Levene's test, and when necessary, the data were log-transformed. Clear outlier values of metal content (three Cu values and two Zn values) in earthworms from the exposure experiment were removed from the data set (these outliers were attributed to human error during measurement).

One-way ANOVA was used to estimate the differences in metallothionein expression and metal content between earthworms from the contaminated and uncontaminated sites. Three-way ANOVA was used for data from the exposure experiment (factors were site, exposure concentration and exposure time). In these analyses, when significant differences were found, the data were split and studied further with two-way or one-way ANOVA. The LSD post hoc test was applied when there was a significant result in ANOVA. The relationship between metal content

Table 3 Differences between *D.octaedra* populations collected at sites differing in metal contamination

	df	SS	MS	*F*	P
Metallothionein	1	227.5	227.5	83.6	<0.001
Cu body content	1	275.3	275.3	4.8	0.032
Zn body content	1	542.5	542.5	19.3	<0.001
Cd body content	1	–	–	50.8 (χ^2)	<0.001

Differences in metallothionein expression and Cu and Zn body contents were analyzed with one-way ANOVA. Difference in Cd body content was analyzed with a Kruskall-Wallis test (χ^2)

of the earthworms and metallothionein expression was studied with Pearson product-moment correlation. When the assumptions of a normal distribution or equal variances were not met even after data transformation, non-parametric methods were used: the Kruskall-Wallis test was used instead of one-way ANOVA and Spearman's rank-order correlation was used instead of Pearson product-moment correlation. All statistics were performed using IBM Statistics SPSS 20.

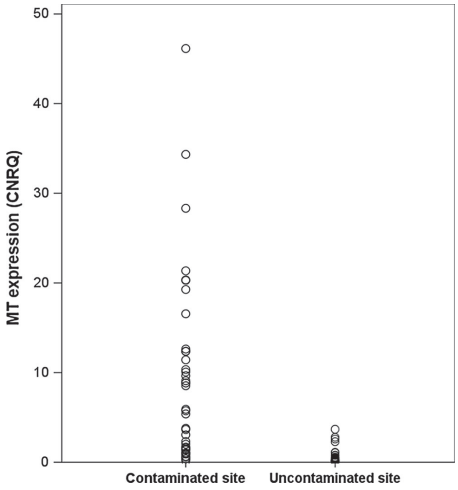


Fig. 1 Metallothionein (MT) expression (CNRQ, calibrated normalized relative quantity, from qBase+) of *D. octaedra* individuals from the contaminated site (Harjavalta), and the uncontaminated site (Jyväskylä)

Results

Population comparison

Dendrobaena octaedra earthworms from the contaminated site (Harjavalta) and the uncontaminated site (Jyväskylä) differed in metallothionein expression and in body content of Cu, Zn and Cd (Table 3). Metallothionein expression was higher in earthworms from the contaminated site than in earthworms from the uncontaminated site (Table 4; Fig. 1). Zn and Cd body content was higher in earthworms from the contaminated site than in the uncontaminated site, but the pattern was reversed for Cu body content (Table 4). Body content of some metals, e.g. Pb, Ni and Cr, were under the limit of quantification (LOQ). Metallothionein expression correlated positively with Zn, Cu and Cd body content in the earthworms collected from the uncontaminated site (Table 4). In contrast, no correlations were found between metallothionein expression and metal body content in the earthworms from the contaminated site (Table 4).

We allowed the earthworms to empty their guts for 2 days so that the metal body content measurements would not be affected by gut content. However, the gut emptying period could have affected metallothionein expression.

Table 4 Means and correlations between metallothionein expression (MT) and metal body content of *D. octaedra* from the contaminated and uncontaminated sites

Test	Site		MT	Cu	Zn	Mean[a]
Pearson	Contaminated	MT	–			8.7
		Cu	0.126 (0.425)	–		69.9
		Zn	0.097 (0.541)	−0.620 (0.696)	–	439
	Uncontaminated	MT	–			0.5
		Cu	0.333(0.033)	–		93.8
		Zn	0.368 (0.018)	0.122 (0.446)	–	324
Spearman	Contaminated	Cd	0.213 (0.176)	0.005 (0.974)	0.713 (<0.001)	19.7
	Uncontaminated	Cd	0.424 (0.025)	0.204 (0.287)	0.609 (<0.001)	3.52

p value in parenthesis

[a] Mean metallothionein expression (*CNRQ* calibrated normalized relative quantity) and mean metal body content (mg/kg dry mass)of the earthworms

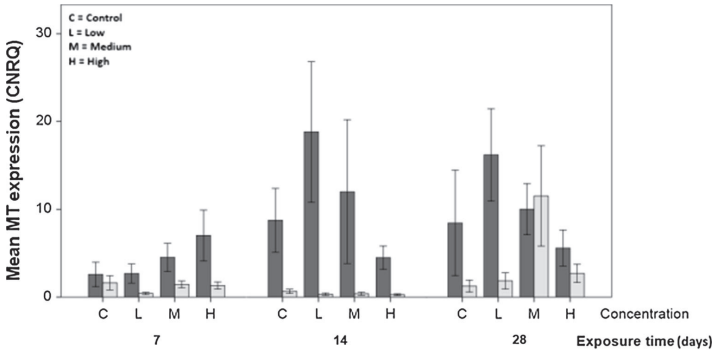


Fig. 2 Metallothionein expression (normalized relative quantity from qBase+, mean ± S.E.) of *D. octaedra* earthworms from contaminated (Harjavalta, *dark grey bars*) and uncontaminated (Jyväskylä, *light grey bars*) sites exposed to *three* different concentrations (*L* low, *M* medium and *H* high) of Cu and control (C) for 7, 14 or 28 days

Consequently, we tested metallothionein expression in an additional sample of 10 individuals from both populations from which RNA was extracted immediately, without gut emptying. The expression levels did not differ between treatments in earthworms from the same population, but significant differences between populations remained (data not shown).

Exposure experiment

Final Cu and Zn concentrations of the test soil used in the exposure experiment were approximately what we intended (Online Resource 1, Table 1) and metal concentrations in the test soils measured before and after the experiment did not differ significantly (Kruskall-Wallis, $p > 0.05$).

Metallothionein response in Cu exposed earthworms

When exposed to Cu, earthworms from the two sites differed in their metallothionein expression ($F = 47.5$, $df = 1$, $p < 0.001$, Fig. 2), there was an interaction between site and exposure time ($F = 5.83$, $df = 2$, $p = 0.005$, Online Resource 1, Table 2), and earthworms from the contaminated site had higher metallothionein expression (mean 8.5 CNRQ over all exposure concentrations and times) than earthworms from the uncontaminated site (mean 1.9 CNRQ). Metallothionein expression was lower in earthworms exposed for shorter periods (7 and 14 days) than in those exposed for 28 days (Online Resource 1, Table 3). Metallothionein expression was higher in earthworms from the contaminated site than in earthworms from the uncontaminated site after 7 and 14 days of exposure, but not after 28 days, although the difference was almost significant ($p = 0.055$) (Online Resource 1, Table 4).

Metallothionein expression was also analyzed separately for earthworms from the two sites with exposure time and soil Cu concentration as factors. The difference in metallothionein expression between exposure times was found in earthworms from the uncontaminated site, but not in earthworms from the contaminated site (Online Resource 1, Table 5). In earthworms from the uncontaminated site, metallothionein expression was highest after 28 days and lowest after 14 days (Online Resource 1, Table 6). The pattern was seen only in the medium and high soil Cu concentrations, although there was no significant interaction between exposure time and soil Cu concentration (Online Resource 1, Tables 7 and 8). Two-way ANOVA with soil concentration levels separated and site and exposure time as factors showed that metallothionein expression was higher in earthworms from the contaminated site at every exposure level, and also in control earthworms (Online Resource 1, Tables 9 and 10).

Metallothionein response in Zn exposed earthworms

Earthworms from the two sites exposed to Zn differed in metallothionein expression ($F = 56.87$, $df = 1$, $p < 0.001$, Fig. 3). Earthworms from the contaminated site had higher metallothionein expression (mean: 3.6 CNRQ) than earthworms from the uncontaminated site (mean: 0.6 CNRQ).Differences were also detected among the exposure concentrations (control, low, medium or high, $F = 7.112$, $df = 3, p < 0.001$) as well as among the exposure times (7, 14 or 28 days, $F = 7.642$, $df = 2, p = 0.001$, Online Resource 1, Table 11). Earthworms exposed for 28 days expressed more metallothionein than earthworms exposed for 7 or 14 days, and control earthworms expressed more metallothionein than earthworms from any of the contamination levels (Online Resource 1, Tables 12 and 13).

Although there were no significant interactions between the factors in the three-way ANOVA, we examined the data further with two-way ANOVA. Analysis with site and

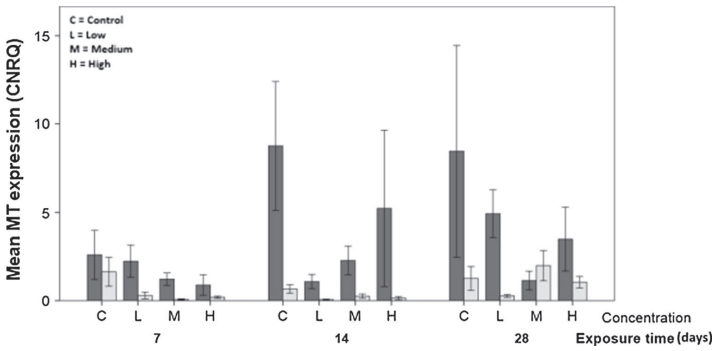


Fig. 3 Metallothionein expression (normalized relative quantity from qBase+, mean ± S.E.) of *D. octaedra* earthworms from contaminated (*dark grey bars*, Harjavalta) and uncontaminated (*light grey*, Jyväskylä) sites exposed to *three* different concentrations (*L* low, *M* medium and *H* high) of Zn and control (C) for 7, 14 or 28 days

exposure concentration as factors showed that metallothionein expression differed between earthworms from the two sites at each exposure time (7, 14 or 28 days of exposure), as well as between different exposure concentrations after 7 days and 14 days of exposure (Online Resource 1, Tables 14 and 15). In the two-way ANOVA there was an interaction between site and concentration after 28 days of exposure (Online Resource 1, Table 14). Two-way ANOVA for each site separately showed that in earthworms from the uncontaminated site, there was also a difference between exposure times (lower expression with shorter exposure) and an interaction between exposure time and concentration (Online Resource 1, Table 16, 17 and 18).

One-way ANOVA with exposure time as a factor showed that the variation in metallothionein expression between exposure times in earthworms from the contaminated site was in the low exposure treatment of Zn (Online Resource 1, Table 19). In earthworms from the uncontaminated site, in contrast, the difference in expression at different times was in earthworms exposed to medium and high levels of Zn (Online Resource 1, Table 19).

Metal content of earthworms

The accuracy of the analysis was tested with the analysis of CRM DOLT-4 (Dogfish liver). The recovery rates for Cu and Zn were 100 ± 3 % and 102 ± 2 %, respectively. Earthworms exposed to Cu from the two sites differed in Cu content ($F = 4.552$, $df = 1$, $p = 0.036$, Online Resource 1, Table 20, see also Fig. 4). Earthworms from the uncontaminated site had higher Cu content (mean: 116.1 mg/kg) than earthworms from the contaminated site (mean: 95.6 mg/kg). Two-way ANOVA with exposure concentrations separated and site and exposure time as factors indicated that earthworms from the two sites differed in Cu content only in control earthworms ($F = 5.296$, $df = 1$, $p = 0.034$). Moreover, two way ANOVA with exposure times separated and site and exposure concentration as factors showed that Cu content of the earthworms differed between the two sites in earthworms exposed for 7 days only ($F = 11.205$, $df = 1$, $p = 0.003$). No differences between sites, concentrations or exposure time were found in Zn content of earthworms exposed to Zn (Online Resource 1, Table 21, see also Fig. 5). There was a positive correlation between metallothionein expression and Zn content of the earthworms in Zn exposed earthworms from the contaminated site (Pearson correlation, $r = 0.418$, $p = 0.003$). No correlations were found between Cu body content and metallothionein expression of the experimentally exposed earthworms.

Discussion

Dendrobaena octaedra are able to adapt to metal exposure (Fisker et al. 2011a, 2011b, 2013). It is important to assess how possible adaptation could affect the expression of metallothionein, a well-characterized biomarker used in measuring organismal response to metal exposure (e.g. Dallinger 1996; Brulle et al. 2007, 2011; Carpene et al. 2007). We found that metallothionein-2 (mt-2) expression is higher in earthworms originating from a population with metal exposure history than in those without metal exposure history. In our exposure experiment, earthworms from the contaminated site had stable, high metallothionein expression regardless of exposure concentration or exposure time. In contrast, mt-2 expression increased with increasing exposure time in earthworms originating from the uncontaminated site. We did not find a consistent dose–response of expression in either population, indicating that the essential metals tested here, Cu and Zn, are not as efficient inducers of this isoform of metallothionein as e.g. Cd for which dose–response is often found (Spurgeon et al.

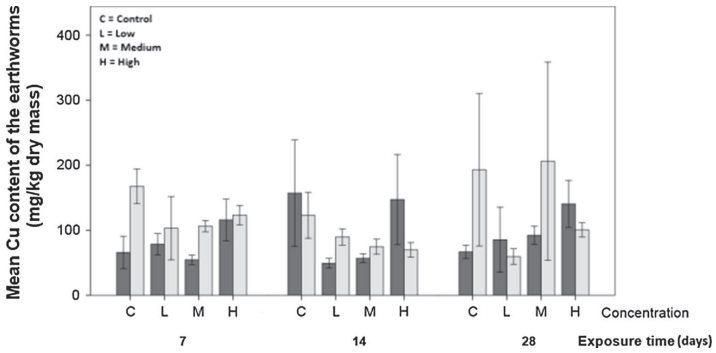


Fig. 4 Cu content (mean ± S.E.) of *D. octaedra* earthworms from contaminated (Harjavalta, *dark grey bars*) and uncontaminated (Jyväskylä, *light grey bars*) sites exposed to *three* different concentrations (*L* low, *M* medium and *H* high) of Cu and control (C) for 7, 14 or 28 days

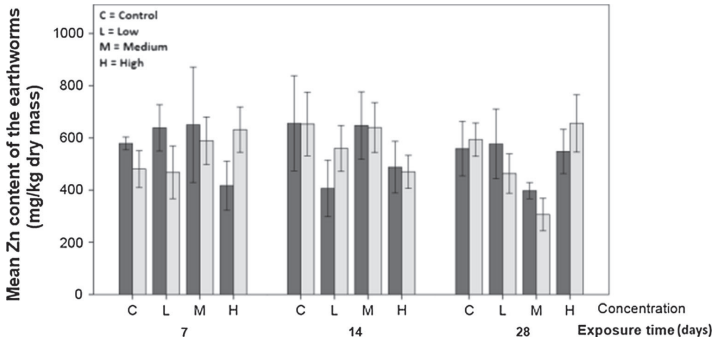


Fig. 5 Zn content (mean ± S.E.) of *D. octaedra* earthworms from contaminated (Harjavalta, *dark grey bars*) and uncontaminated (Jyväskylä, *light grey bars*) sites exposed to *three* different concentrations (*L* low, *M* medium and *H* high) of Zn and control (C) for 7, 14 or 28 days

2004, 2005b; Swain et al. 2004; Galay Burgos et al. 2005; Timmermans et al. 2005; Brulle et al. 2007).

Adaptation to metal exposure is known to occur in soil invertebrates (Kozlov and Zvereva 2011; Pauwels et al. 2013) and changes in metallothionein expression, including over-expression and quick expression responses upon metal exposure, are associated with adaptation, e.g. in the collembolan *Orchesella cincta* (Janssens et al. 2009; Roelofs et al. 2008, 2009) and the earthworm *A. tuberculata* (Lukkari et al. 2004a). On the other hand, studies with *L. rubellus* showed that acclimatization is a more likely explanation for its metal tolerance than is adaptation (Aziz et al. 1999; Langdon et al. 2003). It is important to remember that different populations could show different strategies of metal tolerance (Pauwels et al. 2013), but since genetic adaptation to Cu exposure has been demonstrated in *D. octaedra* (Fisker et al. 2013), we expect that other populations with a history of Cu exposure could also adapt. Therefore, the mt-2 response of the populations studied here could reflect not only acclimatization (in the uncontaminated site), but also the combination of acclimatization and potential adaptation (in the contaminated site).

We found considerable variation among individuals in metallothionein expression, both in our comparison of samples from the field and in our exposure experiment. In earthworms from the field, this variation could reflect the avoidance behavior of *D. octaedra* (Lukkari and Haimi 2005), since in the contaminated site, metals are not evenly distributed in the soil (Salminen and Haimi 1999), and earthworms avoid the most contaminated soil patches. Consequently, inter-individual exposure to metals varies, which might explain variation in mt-2 expression. However, there was also high inter-individual variation in mt-2 expression among worms from the uncontaminated site and in our experiment (where there was no need or no opportunity for avoidance). High genetic diversity of *D. octaedra* (Simonsen et al. 2008; Knott and Haimi 2010) could also explain the high inter-individual variation in metallothionein expression. Recently, (Nota et al. 2013) showed that in the parthenogenetic *Folsomia candida,* two genotypes within a previously suspected clonal line have large

differences in their response to Cd, one being more tolerant and other more sensitive. In addition, high genetic diversity is complicated by polyploidy in *D. octaedra*, which is usually hexaploid (Omodeo 1955; Hongell and Terhivuo 1989). If some individuals have multiple functional copies of the metallothionein gene, they could show higher expression than other individuals with fewer copies. The possibility for inter-individual variation in metallothionein gene copy number and its relationship to expression differences in *D. octaedra,* however, remains to be investigated.

In our experiment, exposure time affected only the earthworms from the uncontaminated site, suggesting that, in addition to overall lower expression, there was also a slower response in metallothionein induction in these earthworms than in those from the contaminated site. A similar result was found by Fisker and colleagues (2013), who did not see upregulation of mt-2 expression after 1 day of Cu exposure, but did after 7 days of exposure in *D. octaedra* from a reference population. In our study, upregulation was clearest after 28 days of exposure, suggesting that a longer exposure period improves the chances of observing an acclimatization response in these earthworms. The time response in earthworms from the uncontaminated site was found only in the medium and high exposure levels, suggesting that equilibrium between soil and earthworm body metal content was achieved faster at low metal concentrations. When equilibrium is achieved, longer exposure times are not expected to lead to higher metallothionein expression, since additional expression is not needed. Equilibrium is a plausible explanation for our results, since a pattern of stable mt-2 expression at low metal concentrations with longer exposure time was seen with both tested metals. Maity et al. (2011) made similar conclusions for metallothionein expression in the earthworm *Lampito mauritii* exposed to Zn. Cu and Zn are both essential metals, and at low soil metal concentration, equilibrium between uptake and excretion of the metals can be achieved relatively quickly, unlike for nonessential metals, which tend to accumulate in the tissues (Spurgeon and Hopkin 1999).

In contrast to the time-response, we did not detect a consistent dose–response of metallothionein-2 expression in our experiment. Cu exposed earthworms showed no signs of a dose related response, and in Zn exposed earthworms, the only significant effects of dose on metallothionein expression were due to a difference between exposed and control samples, not between different metal exposures. Among soil invertebrates, a dose–response in metallothionein induction to the nonessential metal Cd has been found frequently (Spurgeon et al. 2004, 2005b; Swain et al. 2004; Galay Burgos et al. 2005; Timmermans et al. 2005; Brulle et al. 2007), but not always (Demuynck et al. 2007). The opposite has been shown for Cu or Zn: clear dose–responses are more often not found, with a few exceptions (Spurgeon et al. 2005b; Brulle et al. 2007; Bundy et al. 2008). For example, in *L. rubellus,* Galay Burgos et al. (2005) found increased metallothionein expression at low Cu concentrations and decreased expression at high Cu concentrations, and Demuynck et al. (2007) found no dose–responseof *E. fetida* to Zn and Cd. Cu and Zn are not expected to be as efficient inducers of metallothionein as is Cd (Marino et al. 1998; Swain et al. 2004; Brulle et al. 2011), and thus it may not be surprising that we did not find a clear dose–response in our experiments. The fact that we allowed earthworms to empty their gut prior to measuring mt-2 expression may have affected the results and obscured a possible dose-response. However, a small sample of earthworms from the study sites showed no difference in mt-2 expression between treatments (with and without gut emptying).

An additional reason for the lack of dose-response in earthworms from the contaminated site could be potential adaptation. The fact that metallothionein expression was higher in earthworms from the contaminated site even when they were not exposed to any added metal (i.e. control earthworms) and was similarly high in all exposure concentrations supports this interpretation. Adaptation can lead to over-expression of stress-response genes (Roelofs et al. 2008), which could mean that in comparative analysis of gene expression, individuals from adapted populations might show no response or only a small response to increasing concentrations of metals (Pauwels et al. 2013). Therefore, it is important to know the history of the test population and its potential for adaptation to metal exposure when conducting ecotoxicological experiments. On the other hand, Nota et al. (2013) showed that there can be differences in the stress response (dose-responses vs. stable expression levels) even among different genotypes of *F. candida* originating from a population without exposure history. Moreover, dose-responses are known for individuals originating from populations with exposure history e.g. in *A. tuberculata* (Lukkari et al. 2004a) as well as in our study species *D. octaedra* (Fisker et al. 2013). Whether or not inducibility of a stress response is lost in populations with exposure history could, therefore, be context dependent and requires further study.

Metallothionein expression does not seem to be directly related to metal body content. Among field collected earthworms, metallothionein expression was positively correlated with Cu, Zn and Cd body content in samples from the uncontaminated site but not the contaminated site. This could be an artefact of the overall high mt-2 expression in earthworms from the contaminated site and also could be the result of adaptation. In the exposure experiment, metal body content was similar in earthworms from

both sites, but there was a difference in metallothionein expression. The small sample size and possibility that other metal-binding proteins or other isoforms of metallothionein influence metal body content complicates the interpretation of these correlation analyses.

Although Cu soil concentration was not correlated with Cu body content in the worms, the result was nearly significant ($p = 0.05$). Cu typically does not bioaccumulate in tissues over the concentrations found in the soil, but high exposure levels do elevate body content (Marino et al. 1998). As a result, even though Cu is an essential metal and is regulated (Laszczyca et al. 2004), a relationship is often found between Cu body content and soil Cu concentration (Lukkari et al. 2006; Galay Burgos et al. 2005; Spurgeon et al. 2005b; Holmstrup et al. 2011), as opposed to Zn (Lukkari et al. 2006; Demuynck et al. 2007; Brulle et al. 2011; Holmstrup et al. 2011; Leveque et al. 2013). Since the Cu body content of the earthworms differed between the sites only between control earthworms and after 7 days of exposure, this could be evidence of a legacy from their life in the field. Cu body content of earthworms from the uncontaminated site were quite high (mean 93.8 mg/kg dry weight), considering the amount of Cu in the soil. But these values were still within the range reported in a previous study with this species (Lukkari et al. 2004a), in which even higher Cu body contents were found. The higher Cu content of earthworms from the uncontaminated site could be due to less efficient excretion or increased uptake of Cu, and possibly, uptake of Cu from multiple metal pools (Hobbelen et al. 2006).

In conclusion, populations differing in their exposure to metals showed different metallothionein expression, in both a comparison of samples collected from the field and an exposure experiment. The potential for adaptation to metal exposure in the population from the contaminated site is a plausible explanation for these differences. Metallothioneins can be induced by factors other than metals also (Janssens et al. 2009), such as oxidative stress and hypoxia, and these cannot be ruled out as explanations for the differences in expression seen here. Nevertheless, there was a difference in metallothionein expression between earthworms from the two sites also in our exposure experiment, during which the earthworms were exposed to the same conditions and novel soil. Overall high metallothionein expression could be one outcome of potential adaptation. The increased expression of metallothionein is expected to aid detoxification and regulation of metals, making it possible for earthworms to cope in a metal contaminated environment. In addition, adaptation could lead to the loss of an inducible response to metal exposure, as was suggested by results of our exposure experiment in which earthworms from the contaminated site expressed metallothionein at high and stable levels regardless of the exposure concentration or exposure time, and even when there was no exposure.

Acknowledgments This work was supported by the Maj and Tor Nessling Foundation (Project Number 2014140). The authors are grateful to Jasmin Nevala and Jemina Nevala for assistance in the field and lab. Experiments performed in this study comply with the current laws of Finland.

Conflict of interest The authors declare that they have no conflict of interest.

References

Asensio V, Rodriguez-Ruiz A, Garmendia L, Andre J, Kille P, Morgan AJ, Soto M, Marigomez I (2013) Towards an integrative soil health assessment strategy: a three tier (integrative biomarker response) approach with *Eisenia fetida* applied to soils subjected to chronic metal pollution. Sci Total Environ 442:344–365

Aziz NA, Morgan AJ, Kille P (1999) Metal resistance in earthworms; genetic adaptation or physiological acclimation. Pedobiologia 43(6):594–601

Bengtsson G, Nordstrom S, Rundgren S (1983) Population-density and tissue metal concentration of lumbricids in forest soils near a brass mill. Environ Pollut Ser A Ecol Biol 30:87–108

Bengtsson G, Ek H, Rundgren S (1992) Evolutionary response of earthworms to long-term metal exposure. Oikos 63:289–297

Bindesbøl AM, Bayley M, Damgaard C, Hedlund K, Holmstrup M (2009) Changes in membrane phospholipids as a mechanistic explanation for decreased freeze tolerance in earthworms exposed to sublethal copper concentrations. Environ Sci Technol 43:5495–5500

Bradl H (2005) In: Hubbard A (ed) Heavy metals in the environment: origin, interaction and remediation. interface science and technology. Elsevier, Neubrucke

Brulle F, Mitta G, Cocquerelle C, Vieau D, Lemiere S, Lepretre A, Vandenbulcke F (2006) Cloning and real-time PCR testing of 14 potential biomarkers in *Eisenia fetida* following cadmium exposure. Environ Sci Technol 40:2844–2850

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? Comp Biochem Physiol C 144:334–341

Brulle F, Lemiere S, Waterlot C, Doyay F, Vandenbulcke F (2011) Gene expression analysis of 4 biomarker candidates in *Eisenia fetida* exposed to an environmental metallic trace element gradient: a microcosm study. Sci Total Environ 409:5470–5482

Bundy J, Sidhu J, Rana F, Spurgeon D, Svendsen C, Wren J, Sturzenbaum S, Morgan 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

Calisi A, Zaccarelli N, Lionetto MG, Schettino T (2013) Integrated biomarker analysis in the earthworm *Lumbricus terrestris*: application to the monitoring of soil heavy metal pollution. Chemosphere 90:2637–2644

Carpene E, Andreani G, Isani G (2007) Metallothionein functions and structural characteristics. J Trace Elem Med Biol 21:35–39

Cortet J, Gomot-De-Vauflery A, Gomot L, Poinsot-Balaguer N, Texier C, Cluzeau D (1999) The use of invertebrate soil fauna in monitoring pollutant effects. Eur J Soil Biol 35:115–134

Dallinger R (1996) Metallothionein research in terrestrial invertebrates: synopsis and perspectives. Comp Biochem Physiol C 113:125–133

Demuynck S, Grumiaux F, Mottier V, Schikorski D, Lemiere S, Lepretre A (2006) Metallothionein response following cadmium exposure in the oligochaete *Eisenia fetida*. Comp Biochem Physiol C 144:34–46

Demuynck S, Grumiaux F, Mottier V, Schikorski D, Lemiere S, Lepretre A (2007) Cd/Zn exposure interactions on metallothionein response in *Eisenia fetida* (*Annelida*, *Oligochaeta*). Comp Biochem Physiol C 145:658–668

Edwards CA, Bohlen PJ (1996) Biology and ecology of earthworms, 3rd edn. Chapman & Hall, London

Fisker KV, Sørensen JG, Damgaard C, Pedersen KL, Holmstrup M (2011a) Genetic adaptation of earthworms to copper pollution: is adaptation associated with fitness costs in *Dendrobaena octaedra*? Ecotoxicology 20:563–573

Fisker KV, Sørensen JG, Holmstrup M (2011b) No costs on freeze tolerance in genetically copper adapted earthworm populations (*Dendrobaena octaedra*). Comp Biochem Physiol C 154:204–207

Fisker KV, Holmstrup M, Sørensen JG (2013) Variation in metallothionein gene expression is associated with adaptation to copper in the earthworm *Dendrobaena octaedra*. Comp Biochem Physiol C 157:220–226

Galay Burgos M, Winters C, Sturzenbaum S, Randerson P, Kille P, Morgan J (2005) Cu and Cd effects on the earthworm *Lumbricus rubellu*s in the laboratory: multivariate analysis of relationships between exposure, biomarkers, and ecologically relevant parameters. Environ Sci Techol 39:1757–1763

Gruber C, Sturzenbaum S, Gehrig P, Sack R, Hunziker P, Berger B, Dallinger R (2000) Isolation and characterization of a self-sufficient one-domain protein—(Cd)-metallothionein from *Eisenia fetida*. Eur J Biochem 267:573–582

Hobbelen PHF, Koolhaas JE, van Gestel CAM (2006) Bioaccumulation of heavy metals in the earthworms *Lumbricus rubellus* and *Aporrectodea caliginosa* in relation to total and available metal concentrations in field soils. Environ Pollut 144:639–646

Holmstrup M, Sørensen JG, Overgaard J, Bayley M, Bindesbøl AM, Slotsbo S, Fisker KV, 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

Hongell K, Terhivuo J (1989) Chromosomal status of the parthenogenetic earthworm *Dendrobaena octaedra* (Sav) (*Oligochaete*, *Lumbricidae*) in southern Finland. Hereditas 110(2):179–182

Hooper DU, Adair EC, Cardinale BJ, Byrnes JEK, Hungate BA, Matulich KL, Gonzales A, Duffy JE, Gamfeldt L, O'Connor MI (2012) A global synthesis reveals biodiversity loss as a major driver of ecosystem change. Nature 486:105–108

Janssens TKS, Roelofs D, van Straalen NM (2009) Molecular mechanisms of heavy metal tolerance and evolution in invertebrates. Insect Sci 16:3–18

Knott KE, Haimi J (2010) High mitochondrial DNA sequence diversity in the parthenogenetic earthworm *Dendrobaena octaedra*. Heredity 105:341–347

Kozlov MV, Zvereva EL (2011) A second life for old data: global patterns in pollution ecology revealed from published observational studies. Environ Pollut 159:1067–1075

Langdon CJ, Piearse TG, Meharg AA, Semple KT (2003) Inherited resistance to arsenate toxicity in two populations of *Lumbricus rubellus*. Environ Toxicol Chem 22(10):2344–2348

Laszczyca P, Augustyniak M, Babcrynska 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). Environ Int 30:901–910

Lavelle P, Decaens T, Aubert M, Barot S, Blouin M, Bureau F, Margerie P, Mora P, Rossi J-P (2006) Soil invertebrates and ecosystem services. Eur J Soil Biol 42:S3–S15

Leveque T, Capowiez Y, Schreck E, Mazzia C, Auffan M, Foucault Y, Austruy A, Dumat C (2013) Assessing ecotoxicity and uptake of metals and metalloids in relation to two different earthworm species (*Eisenia hortensis* and *Lumbricus terrestris*). Environ Pollut 179:232–241

Lukkari T, Haimi J (2005) Avoidance of Cu- and Zn-contaminated soil by three ecologically different earthworm species. Ecotoxicol Environ Saf 62:35–41

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

Lukkari T, Taavitsainen M, Väisänen A, Haimi J (2004b) Effects of heavy metals on earthworms along contamination gradients in organic rich soils. Ecotox Environ Safe 59:340–348

Lukkari T, Teno S, Väisänen A, Haimi J (2006) Effects of earthworms on decomposition and metal availability in contaminated soil: microcosm studies of populations with different exposure histories. Soil Biol Biochem 38:359–370

Maity S, Roy S, Bhattacharya S, Chaudhury S (2011) Metallothionein responses in the earthworm *Lampito mauritii* (Kinberg) following lead and zinc exposure: a promising tool for monitoring metal contamination. Eur J Soil Biol 47:69–71

Marino F, Sturzenbaum SR, Kille P, Morgan AJ (1998) Cu–Cd interactions in earthworms maintained in laboratory microcosms: the examination of a putative copper paradox. Comp Biochem Physiol C 120:217–223

Nahmani J, Hodson ME, Black S (2007) A review of studies performed to assess metal uptake by earthworms. Environ Pollut 145:402–424

Nota B, de Korte M, Ylstra B, van Straalen NM, Roelofs D (2013) Genetic variation in parthenogenetic collembolans is associated with differences in fitness and cadmium-induced transcriptome. Environ Sci Technol 47:1155–1162

Omodeo P (1955) Cariologia dei Lumbricidae II Contributo. Caryologia 8:135–178

Pauwels M, Frenot H, Souleman D, Vandenbulcke F (2013) Using biomarkers in an evolutionary context: lessons from the analysis of biological responses of oligochaete annelids to metal exposure. Environ Pollut 179:343–350

Posthuma L, van Straalen NM (1993) Heavy-metal adaptation in terrestrial invertebrates: a review of occurrence, genetics, physiology and ecological consequences. Comp Biochem Physiol C 106:11–38

Roelofs D, Aarts MGM, Schat H, van Straalen NM (2008) Functional ecological genomics to demonstrate general and specific responses to abiotic stress. Funct Ecol 22:8–18

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

Rozen A (2003) Do earthworms (*Dendrobaena octaedra*) from differently polluted forests vary in life history parameters? Eur J Soil Biol 39:73–77

Rozen A (2006) Effect of cadmium on life-history parameters in *Dendrobaena octaedra* (*Lumbricidae*: *oligochaeta*) populations originating from forests differently polluted with heavy metals. Soil Biol Biochem 38:489–503

Salemaa M (2003) Responses of the understory vegetation of boreal forests to heavy metal loading. Dissertation, University of Helsinki

Salminen J, Haimi J (1999) Horizontal distribution of copper, nickel and enchytraeid worms in polluted soil. Environ Pollut 104:351–358

Simonsen V, Laskowski R, Bayley M, Holmstrup M (2008) Low impact of metal pollution on genetic variation in the earthworm *Dendrobaena octaedra* measured by allozymes. Pedobiologia 52:51–60

Spurgeon DJ, Hopkin SP (1999) Tolerance to zinc in populations of the earthworm *Lumbricus rubellus* from uncontaminated and metal-contaminated ecosystems. Arch Environ Contam Toxicol 37:332–337

Spurgeon DJ, Weeks JM, van Gestel CAM (2003) A summary of eleven years progress in earthworm ecotoxicology. Pedobiologia 47:588–606

Spurgeon DJ, Sturzenbaum SR, Svendsen C, Hankard PK, Morgan AJ, Weeks JM, Kille P (2004) Toxicological, cellular and gene expression responses in earthworms exposed to copper and cadmium. Comp Biochem Physiol C 138:11–21

Spurgeon DJ, Ricketts H, Svendsen C, Morgan AJ, Kille P (2005a) Hierarchical responses of soil invertebrates (earthworms) to toxic metal stress. Environ Sci Technol 39:5327–5334

Spurgeon DJ, Svendsen C, Lister LJ, Hankard PK, Kille P (2005b) Earthworm responses to Cd and Cu under fluctuating environmental conditions: a comparison with results from laboratory exposures. Environ Pollut 136:443–452

Sturzenbaum SR, Kille P, Morgan AJ (1998) The identification, cloning and characterization of earthworm metallothionein. FEBS Lett 431:437–442

Sturzenbaum SR, Andre J, Kille P, Morgan AJ (2009) Earthworm genomes, genes and proteins: the (re)discovery of Darwin's worms. Proc R Soc B 276:789–797

Swain SC, Keusekotten K, Baumeister R, Sturzenbaum SR (2004) *C. elegans* metallothioneins: new insights into the phenotypic effects of cadmium toxicosis. J Mol Biol 341:951–959

Terhivuo J, Saura A (2006) Dispersal and clonal diversity of North-European parthenogenetic earthworms. Biol Invasions 8:1205–1218

Timmermans MJTN, Ellers J, Roelofs D, van Straalen NM (2005) Metallothionein mRNA expression and cadmium tolerance in metal-stressed and reference populations of the springtail *Orchesella cincta*. Ecotoxicology 14:727–739

Tosza E, Dumnicka E, Niklinska M, Rozen A (2010) Enchytraeid and earthworm communities along a pollution gradient near Olkusz (southern Poland). Eur J Soil Biol 46:218–224

Valko M, Morris H, Cronin MTD (2005) Metals, toxicity and oxidative stress. Curr Med Chem 12:1161–1208

Weeks JM, Spurgeon DJ, Svedsen C, Hankard PK, Kammenga JE, Dallinger R, Köhler H, Simonsen V, Scott-Fordsmand J (2004) Critical analysis of soil invertebrate biomarkers: a field case study in Avonmouth, UK. Ecotoxicology 13:817–822

Online Resource 1.

Metallothionein gene expression differs in earthworm populations with different exposure history

Journal: Ecotoxicology

Mustonen, M.,[a] Haimi, J.,[a] Väisänen, A.[b] and Knott, K. E.[a]

[a]Department of Biological and Environmental Science, University of Jyväskylä, P.O. Box 35, 40014, Jyväskylä, Finland
[b]Department of Chemistry, University of Jyväskylä, P.O. Box 35, 40014, Jyväskylä, Finland

Email of corresponding author: marina.s.mustonen@jyu.fi

Table 1. The intended and measured concentrations of Cu and Zn in soil used in the exposure experiments.

Treatment*	Intended concentration mg/kg	Measured concentration mg/kg**
Cu low	50	56.42
Cu medium	100	118.5
Cu high	200	181.9
Zn low	75	131.8
Zn medium	150	172.1
Zn high	300	293.1

* Control soil had mean Cu concentration 9.96mg/kg and mean Zn concentration 42.67mg/kg.

Cu exposed:

Table 2. Effects of site (contaminated vs. uncontaminated), Cu exposure concentration, exposure time and their interactions on metallothionein expression in *D. octaedra* (Three-way ANOVA).

Source of variation	df	SS	MS	*F*	P
Site	1	69.31	69.31	47.51	<0.001
Concentration	3	3.714	1.238	0.849	0.472
Exposure time	2	19.32	9.658	6.620	0.002
Site x Concentration	3	4.127	1.376	0.943	0.425
Site x Exposure time	2	17.00	8.500	5.826	0.005
Concentration x Exposure time	6	9.136	1.523	1.044	0.405
Site x Conc. x Exposure time	6	4.815	0.803	0.550	0.768

Table 3. Pairwise comparisons (post hoc LSD) of exposure times after three-way ANOVA on metallothionein expression of Cu exposed *D. octaedra* earthworms.

I	J	Difference (I-J)	P
7	14	0.4667	0.127

14	28	-1.4295	<0.001
7	28	-0.9628	0.003

Table 4. Effects of site, exposure concentration and their interaction on metallothionein expression of Cu exposed *D. octaedra* earthworms with exposure times separated (Two-way ANOVA).

Days of exposure	Source of variation	df	SS	MS	*F*	P
7	Site	1	12.48	12.48	11.52	0.003
	Concentration	3	3.940	1.313	1.213	0.329
	Site x Concentration	3	0.995	0.332	0.306	0.820
14	Site	1	68.95	68.95	46.10	<0.001
	Concentration	3	0.939	0.313	0.209	0.889
	Site x Concentration	3	2.463	0.821	0.549	0.653
28	Site	1	7.371	7.371	4.114	0.055
	Concentration	3	7.714	2.571	1.435	0.259
	Site x Concentration	3	5.714	1.905	1.063	0.385

Table 5. Effects of Cu exposure concentration, exposure time and their interaction on metallothionein expression of *D. octaedra* earthworms from the contaminated and uncontaminated sites (Two-way ANOVA).

Site	Source of variation	df	SS	MS	*F*	P
Contaminated	Concentration	3	1.614	0.538	0.266	0.849
	Exposure time	2	3.685	1.842	0.910	0.413
	Concentration x Exposure time	6	6.301	1.050	0.519	0.790
Uncontaminated	Concentration	3	6.816	2.272	2.251	0.098
	Exposure time	2	35.53	17.77	17.60	<0.001
	Concentration x Exposure time	6	8.261	1.377	1.364	0.253

Table 6. Pairwise comparisons (post hoc LSD) of exposure times after two-way ANOVA on metallothionein expression of Cu exposed *D. octaedra* earthworms from the uncontaminated site.

I	J	Difference (I-J)	Sig.
7	14	1.0741	0.002
14	28	-2.1657	<0.001
7	28	-1.0915	0.005

Table 7. Effect of exposure time on metallothionein expression of Cu exposed *D. octaedra* earthworms from the uncontaminated site with exposure concentrations separated (One-way ANOVA).

Exposure level	df	SS	MS	F	Sig
Control	2	1.011	0.506	0.378	0.694
Low	2	7.471	3.736	3.734	0.066

Medium	2	24.95	12.47	11.86	0.002
High	2	12.56	6.282	10.40	0.005

Table 8. Pairwise comparisons (post hoc LSD) of exposure times on medium and high treatments after one-way ANOVA on metallothionein expression of Cu exposed *D. octaedra* earthworms from the uncontaminated site.

Exposure level	I	J	Difference (I-J)	Sig.
Medium	7	14	1.6692	0.026
	14	28	-3.3432	0.001
	7	28	-1.6740	0.033
High	7	14	1.3766	0.038
	14	28	-2.3545	0.001
	7	28	-0.9779	0.134

Table 9. Effects of site, exposure time and their interaction on metallothionein expression of Cu exposed *D. octaedra* earthworms with exposure concentrations separated (Two-way ANOVA).

Exposure level	Source of variation	df	SS	MS	*F*	P
Control	Site	1	13.28	13.28	6.671	0.019
	Exposure time	2	0.606	0.303	0.152	0.860
	Site x Exposure time	2	1.297	0.649	0.326	0.726
Low	Site	1	31.05	31.05	26.32	<0.001
	Exposure time	2	9.142	4.571	3.875	0.044
	Site x Exposure time	2	3.798	1.899	1.610	0.233
Medium	Site	1	12.08	12.08	9.296	0.006
	Exposure time	2	16.01	8.005	6.162	0.008
	Site x Exposure time	2	9.246	4.623	3.559	0.048
High	Site	1	14.82	14.82	10.85	0.004
	Exposure time	2	3.786	1.893	1.385	0.276
	Site x Exposure time	2	8.400	4.200	3.074	0.071

Table 10. Pairwise comparisons (post hoc LSD) of exposure times on control, low and medium treatments after two-way ANOVA on metallothionein expression of Cu exposed *D. octaedra* earthworms.

Exposure level	I	J	Difference (I-J)	Sig.
Control	7	14	-0.2726	0.700
	14	28	-0.3491	0.638
	7	28	-0.6217	0.439
Low	7	14	0.2333	0.694
	14	28	-2.0969	0.003
	7	28	-1.8637	0.006
Medium	7	14	0.7514	0.177

	14	28	-2.0901	0.001
	7	28	-1.3387	0.025

Zn exposed:

Table 11. Effects of site, exposure concentration, exposure time and their interactions on metallothionein expression in Zn exposed *D. octaedra* earthworms (Three-way ANOVA).

Source of variation	df	SS	MS	*F*	P
Site	1	82.92	82.92	56.87	<0.001
Concentration	3	31.11	10.37	7.112	<0.001
Exposure time	2	22.28	11.14	7.642	0.001
Site x Concentration	3	8.608	2.869	1.968	0.126
Site x Exposure time	2	7.885	3.943	2.704	0.073
Concentration x Exposure time	6	17.062	2.844	1.950	0.083
Site x Conc. x Exposure time	6	17.216	2.869	1.968	0.080

Table 12. Pairwise comparisons (post hoc LSD) of exposure concentrations after three-way ANOVA on metallothionein expression of Zn exposed *D. octaedra* earthworms.

I	J	Difference (I-J)	Sig.
Control	Low	1.2176	0.001
	Medium	1.3354	<0.001
	High	1.3320	<0.001
Low	Medium	0.1179	0.726
Medium	High	-0.0034	0.992
High	Low	-0.1144	0.726

Table 13. Pairwise comparisons (post hoc LSD) of exposure times after three-way ANOVA on metallothionein expression of Zn exposed *D. octaedra* earthworms.

I	J	Difference (I-J)	Sig.
7	14	-0.3535	0.225
14	28	-0.8830	0.004
7	28	-1.2365	<0.001

Table 14. Effects of site, exposure concentration and their interaction on metallothionein expression of Zn exposed *D. octaedra* earthworms with exposure times separated (Two-way ANOVA).

Exposure time	Source of variation	df	SS	MS	*F*	P
7	Site	1	32.23	32.23	20.00	<0.001
	Concentration	3	17.36	5.787	3.592	0.027
	Site x Concentration	3	7.410	2.470	1.533	0.229
14	Site	1	53.56	53.56	39.73	<0.001
	Concentration	3	29.88	9.959	7.387	0.001

	Site x Concentration	3	1.471	0.490	0.364	0.780
28	Site	1	8.783	8.783	6.182	0.020
	Concentration	3	3.419	1.140	0.802	0.505
	Site x Concentration	3	16.46	5.487	3.863	0.022

Table 15. Pairwise comparisons (post hoc LSD) of exposure concentrations after two-way ANOVA on metallothionein expression of Zn exposed *D. octaedra* earthworms.

Exposure time	I	J	Difference (I-J)	Sig.
7	Control	Low	0.9898	0.134
		Medium	2.0600*	0.003
		High	1.6083*	0.018
	Low	Medium	1.0702	0.085
	Medium	High	-0.4517	0.457
	High	Low	-0.6184	0.311
14	Control	Low	2.0438	0.001
		Medium	0.7658	0.162
		High	2.0238	0.001
	Low	Medium	-1.2781	0.027
	Medium	High	1.2580	0.034
	High	Low	0.0201	0.972

Table 16. Effects of exposure concentration, exposure time and their interaction on metallothionein expression of Zn exposed *D. octaedra* earthworms with sites separated (Two-way ANOVA).

Site	Source of variation	df	SS	MS	*F*	P
Contaminated	Concentration	3	15.01	5.003	3.500	0.025
	Exposure time	2	2.283	1.142	0.799	0.458
	Concentration x Exposure time	6	13.59	2.265	1.584	0.179
Uncontaminated	Concentration	3	26.24	8.747	5.894	0.002
	Exposure time	2	28.01	14.00	9.437	<0.001
	Concentration x Exposure time	6	22.57	3.761	2.535	0.035

Table 17. Pairwise comparisons (post hoc LSD) of exposure concentrations after two-way ANOVA on metallothionein expression of Zn exposed *D. octaedra* earthworms from the uncontaminated site.

I	J	Difference (I-J)	Sig.
Control	Low	1.8435	<0.001
	Medium	1.5532	0.003
	High	1.2831	0.009
Low	Medium	-0.2903	0.548
Medium	High	-0.2701	0.576
High	Low	0.5604	0.231

Table 18. Pairwise comparisons (post hoc LSD) of exposure times after two-way ANOVA on metallothionein expression of Zn exposed *D. octaedra* earthworms from the uncontaminated site.

I	J	Difference (I-J)	Sig.
7	14	0.1277	0.752
14	28	-1.5098	0.001
7	28	-1.3821	0.002

Table 19. Effect of exposure time on metallothionein expression of Zn exposed *D. octaedra* earthworms from the contaminated and uncontaminated site with exposure concentrations separated (One-way ANOVA).

Site	Concentration	df	SS	MS	F	Sig
Contaminated	Control	2	0.748	0.374	0.128	0.882
	Zn 75mg/kg (Low)	2	7.193	3.596	4.624	0.035
	Zn 150mg/kg (Medium)	2	4.345	2.172	2.287	0.157
	Zn 300mg/kg (High)	2	5.145	2.572	1.682	0.235
Uncontaminated	Control	2	1.011	0.506	0.378	0.694
	Zn 75mg/kg (Low)	2	8.709	4.355	3.198	0.080
	Zn 150mg/kg (Medium)	2	27.46	13.73	9.051	0.007
	Zn 300mg/kg (High)	2	13.78	6.891	4.021	0.049

Metal body content of earthworms:

Table 20. Effects of site, exposure concentration, exposure time and their interactions on Cu content in Cu exposed *D. octaedra* earthworms (Three-way ANOVA).

Source of variation	df	SS	MS	*F*	P
Site	1	1.589	1.589	4.552	0.036
Concentration	3	2.862	0.954	2.733	0.050
Exposure time	2	0.221	0.111	0.317	0.729
Site x Concentration	3	2.159	0.720	2.062	0.113
Site x Exposure time	2	1.032	0.516	1.479	0.235
Concentration x Exposure time	6	1.303	0.217	0.622	0.712
Site x Conc. x Exposure time	6	1.885	0.314	0.900	0.500

Table 21. Effects of site, exposure concentration, exposure time and their interactions on Zn content in Zn exposed *D. octaedra* earthworms (Three-way ANOVA).

Source of variation	df	SS	MS	*F*	P
Site	1	0.049	0.049	0.243	0.623
Concentration	3	0.261	0.087	0.434	0.729
Exposure time	2	0.108	0.054	0.270	0.764
Site x Concentration	3	0.436	0.145	0.726	0.539
Site x Exposure time	2	0.266	0.133	0.664	0.518
Concentration x Exposure time	6	1.797	0.300	1.496	0.191
Site x Conc. x Exposure time	6	0.805	0.134	0.670	0.674

II

VARIATION IN GENE EXPRESSION WITHIN CLONES OF THE EARTHWORM *DENDROBAENA OCTAEDRA*

by

Marina Mustonen, Jari Haimi, Jenni Kesäniemi, Harri Högmander & K. Emily Knott

Submitted manuscript

VARIATION IN GENE EXPRESSION WITHIN CLONES OF THE EARTHWORM *DENDROBAENA OCTAEDRA*

Marina Mustonen[1], Jari Haimi[1], Jenni Kesäniemi[2], Harri Högmander[3]

&

K. Emily Knott[1]

[1]Department of Biological and Environmental Science, P.O. Box 35, FI-40014, University of Jyvaskyla, Finland
[2]Department of Ecology, PO Box 3000, FI-90014, University of Oulu, Finland
[3]Department of Mathematics and Statistics, P.O. Box 35, FI-40014, University of Jyvaskyla, Finland

ABSTRACT

Gene expression is highly plastic, which can help organisms to both acclimate and adapt to changing environments. Possible variation in gene expression among individuals with the same genotype (among clones) is not widely considered, even though it could impact the results of studies that focus on gene expression phenotypes, for example studies using clonal lines. We examined the extent of within and between clone variation in gene expression in the earthworm *Dendrobaena octaedra,* which reproduces through apomictic parthenogenesis. Five microsatellite markers were used to confirm that offspring are genetic clones of their parent. After that, expression of 12 genes was measured from five individuals from six different genotypes (collected from two populations) when exposed to altered conditions. A subset of the genes was examined in clonal offspring of two of the genotypes. Gene expression differed between different genotypes, but variation was also high within genotypes. One gene (chymotrypsin inhibitor) showed significant differences in the expression levels among genetically identical individuals. Gene expression can vary considerably, and the extent of variation may depend on the genotypes and genes studied. Ensuring a large sample, with many different genotypes, is critical in studies comparing gene expression phenotypes. Researchers should be especially cautious inferring gene expression phenotypes when using only a single clonal or inbred line, since the results might be specific to only certain genotypes.

INTRODUCTION

Gene expression is regulated at multiple stages (transcriptional and post-transcriptional), and is highly plastic, allowing organisms to both acclimate and adapt to changing environmental conditions [1, 2, 3]. For example, genes related to stress tolerance may show wide variation in expression depending on the conditions organisms experience [e.g. 2, 4, 5]. Genes are expressed differently in different tissues, both during ontogenesis [6] and in fully developed adults [7]. Nowadays, measuring gene expression is relatively easy, allowing examination of gene expression levels as a phenotypic response to altered conditions. Such studies have helped to identify the mechanisms underlying phenotypic changes and to clarify the connection between genotype and phenotype [8, 9]. However, because gene expression can vary, the variation must also be considered when examining gene expression as a phenotypic response. One approach to reduce the possible variation is to examine only a single clonal genotype or nearly identical individuals from inbred lines. An alternative approach is to encompass the possible variation by using pools of different individuals when determining gene expression levels [9].

The amount of variation in gene expression response to identical conditions among individuals sharing the same clonal genotype is rarely reported, even though it is feasible that genotypically identical individuals might not express genes in an identical way. Such variation could be due to physiological differences among the clonal individuals [6, 7] or due to epigenetic differences [10], as shown recently in clonally propagated oil palms [11]. Variation in gene expression has been studied among essentially identical individuals from strains of mice in which the genetic differences have been diminished with many generations of inbreeding [12, 13]. Both of these studies found differences in the gene expression phenotype of individuals that were from the same hybrid line and exposed to the same conditions. Wolff [12] attributed the differences to possible unrecognized variation in the microenvironment. Pritchard and colleagues [13] concluded that there is either tolerance for a wide range of gene expression, or that there could be a selective advantage to having variation in gene expression. Variation in gene expression among individuals from the same clonal genotype might have practical implications for comparative studies that are not widely recognized. Namely, the results of studies focusing on only a single clone or inbred line might not be relevant to other clones or lines, and the results of studies using pools of individuals might mask important variation [9].

An alternative problem also exists: experimental individuals assumed to be clones could actually harbor unexpected genetic differences that affect gene expression. For example, Nota and colleagues [14] found that one of their "clonal" lines of the collembolan *Folsomia candida*, a common test organism for ecotoxicological tests, was actually composed of two genetically different lineages that had surprisingly different expression profiles, with one lineage being much less affected by cadmium exposure than the other. They cautioned that

outcomes of tests using *F. candida* could lead to different interpretations if possible genetic variation in presumed clonal lines is overlooked [14]. Similarly, Dalziel and colleagues [8] advocated using clonal lineages to control for background genetic variation when studying variation in candidate genes, but cautioned that the major challenge for this approach is the possibility for unrecognized additional mutations within the clonal lineage.

This study focuses on gene expression and genetic diversity of the clonally reproducing earthworm, *Dendrobaena octaedra.* This epigeic earthworm is an ecologically important decomposer animal in Northern Boreal forests [15, 16]. *D. octaedra* has high genetic diversity [17, 18, 19, 20] and it is able to adapt to metal contaminated habitats [21, 22, 23]. Our previous study revealed that *D. octaedra* individuals from two populations differing in metal exposure history had significantly different expression of the metallothionein-2 gene and showed different gene expression responses in an exposure experiment [24]. However, we did not specifically assess genotypic diversity in the samples used in that study. Here, we aim to test if gene expression responses differ among individuals sharing the same clonal genotype, originating from the same two populations as in the previous study (uncontaminated and metal contaminated site, Mustonen *et al*. 2014) when they are exposed to altered environmental conditions.

D. octaedra is polyploid and expected to produce clonal offspring through apomictic (mitotic) parthenogenesis [25, 26, 27]. Although a deviation from apomictic parthenogenesis is unlikely, Simonsen and Holmstrup [28] found differences among offspring and their parents in allozyme markers, which led them to question whether some mechanism other than apomictic parthenogenesis can be employed by this species. Although allozyme markers can be used to survey genotypic differences among individuals, their detection is based on the activity of the enzymes and therefore the variation observed can also reflect phenotypic (gene expression) differences among individuals. We developed microsatellite markers for an alternative method to genotype individuals of *D. octaedra*. After determining whether our cultures of *D. octaedra* were reproducing clonally, we used qPCR to examine variation in gene expression both within and among genotypes in two generations of earthworms. We hypothesized that individuals sharing the same genotype would show less variation in gene expression than individuals with different genotypes. It is important to quantify how much variation in gene expression within genotypes exists so that potential causes of the variation, whether they are genetic or epigenetic, can be explored.

MATERIALS AND METHODS

Sampling and establishment of earthworm cultures

Individuals of *Dendrobaena octaedra* were collected from two locations: a metal contaminated site in Harjavalta (South-West Finland, 61°18'50''N, 22°08'30''E)

and an uncontaminated site in Jyväskylä (Central Finland, 62°12'39''N, 25°44'13''E). The source of the metal contamination in Harjavalta is a Cu-Ni smelter that has been operational for decades. Earthworms were collected by hand from both sites in September and October 2012 and transferred to the laboratory in buckets with soil from the sampling site. Cultures were then established in the laboratory. For this study, the population origin of the earthworms was not under our primary focus, but it will be explored in a future experiment.

Juvenile or subadult earthworms were put either singly or in pairs (to allow a possibility for mating) in separate glass jars (Ø 8 cm, with perforated lids) containing uncontaminated organic-rich soil and horse manure (for nutrition). All cultures were maintained at 15 °C in a climate cabinet. Soil was changed every few months. *D. octaedra* has a long generation time (mean maturation time 21.2 months [29]), and we began monitoring the cultures for cocoons (offspring) after approximately 3 months. Part of the single-worm cultures and all pair-worm cultures were used for microsatellite genotyping to determine if the offspring produced in a culture were clones, sharing the same genotype. The number of cocoons produced by the earthworms in each culture varied. Cocoons were removed from the soil about every 14 days, placed on moist paper towel in lidded Petri dishes and reared at 15 °C. Hatching of cocoons was monitored for about 90 days and hatched offspring were collected, frozen with liquid nitrogen and stored at -20 °C for later DNA extraction. Mortality was high, but when possible, the parent earthworm(s) in these cultures were also frozen for later DNA extraction after they had produced about 15 cocoons.

The remaining single worm cultures were used to provide earthworms for the gene expression study. These cultures were maintained as described above, and after about 24 months, five presumably clonal individuals (adult offspring of the wild caught earthworms) were removed from each culture and rehoused separately in Ø 5.5 cm glass jars with perforated lids containing copper contaminated soil for the experiment (see below). An additional earthworm from each culture was used for genotyping to ensure that different genotypes were used in the experiment (Supporting information S1 Table).

For the experiment, we wanted to expose the earthworms to altered conditions that could elicit a gene expression response in the worms, so instead of using the same organic-rich soil used during culturing, we used soil contaminated with copper (100 mg kg^{-1} dry mass). Soil used in the experiment was collected from an uncontaminated mature spruce forest in Jyväskylä (soil water content = 52.5%; pH = 5.3; organic matter content = 82.1%). We mixed 191.3 mg of copper chloride ($CuCl_2$ H_2O) in 1500 ml of water thoroughly with 1.5 kg soil (fresh mass) to achieve the intended contamination level. After addition of the copper chloride, pH of the soil decreased to 5.1 and soil water content increased to 76.3%. The spiked soil was incubated for 7 days at 15 °C before the experiment. During the experiment, water was added to the cultures every other week to replace what was lost due to evaporation. As our goal was not to study the effect of Cu exposure *per se,* we used only a single Cu concentration in the

experiment. Our previous study [24] indicated that this level of Cu contamination was not lethal to *Dendrobaena octaedra*.

Figure 1 shows the experimental setup. The experiment was begun using five individuals per genotype (genotypes reared separately as described above) from six different genotypes, three originating from Jyväskylä (genotypes coded J) and three from Harjavalta (genotypes coded H). These worms were the parent generation in the experiment. The parents were exposed to the contaminated soil for two months, during which they produced cocoons. Cocoons were collected from the experimental cultures, placed on moist paper towel in lidded petri dishes, and maintained at 15 ^{o}C. After the offspring had hatched, they were also reared separately in the Cu contaminated (100 mg kg^{-1} dry mass) soil for two months (the offspring generation). After their respective two month exposure periods, the parent and offspring earthworms were removed from their cultures and put in glass jars with moist paper towel for two days to allow them to empty their guts. Paper towel was changed after one day. A piece of tissue (ca. 5 mm) from the anterior end of each parent earthworm, and about half of each offspring earthworm (also ca. 5 mm) was cut with a scalpel, placed in separate 1.5 ml microcentrifuge tubes and immediately frozen in liquid nitrogen. Samples were stored at -80 °C until RNA extraction (approx. 1-2 months).

Figure 1. Figure showing the experimental setup and which genotypes were used in the analyses from the parent and offspring generations, (in the parent generation, five individuals per genotype were used, except for genotypes H2 and J3, from which there were only three individuals per genotype). Also shown are examples of which samples were used to calculate the different estimates of variation (EV), see section 2.4.1 in Materials and Methods.

Isolation of microsatellite markers

Microsatellite loci were isolated following the FIASCO technique [30] with some modifications [see 31]: the enrichment step included the construction of two microsatellite-enriched libraries using $(GA)_{20}$ and $(CAG)_{11}$ probes. Potential loci were cloned using the pCR2.1-TOPO vector and TOPO-TA cloning kit (Invitrogen) and One Shot TOP10 competent *Escherichia coli* cells. Positive clones were first amplified and sequenced with vector-specific M13 primers. When microsatellite repeats were found in the sequences, new locus specific primers were designed to the flanking regions of the repeat regions using PRIMER3 [32]. Primers were designed for six microsatellite loci, but, after initial testing, one (DO5) did not amplify consistently and was not considered further. The other five loci are described in Table 1.

Genotyping

Genomic DNA was extracted from either whole earthworms (offspring) or a piece of the anterior end (parents) using Qiagen chemistry (DNeasy kit reagents) and a Kingfisher magnetic processor (Thermo Fisher Scientific). Success of the DNA extractions was confirmed with agarose gel electrophoresis. Amplification

was performed separately for each locus in 10 μl reactions containing 1 μl of template DNA, 1X buffer (Biotools), 0.4 mM dNTPs (Fermentas), 1 μM reverse primer, 0.9 μM forward primer (TAG Copenhagen), 0.1 μM labeled forward primer (Applied Biosystems), 2 mM $MgCl_2$ (Biotools) and 0.5 units Taq polymerase (Biotools). Thermocycling conditions were 94 °C for 3 min, then 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, ending with a final extension of 72 °C for 10 min. Amplification products were separated using the ABI PRISM 3130xl with GeneScan 500 LIZ size standard and genotyped using GeneMapper 5 software (all Applied Biosystems).

Gene expression

Our goal was to examine gene expression of each genotype in all of the exposed parents as well as in five offspring each from three parents of each genotype. However, due to some molding of the cocoons, enough offspring hatched only from two of the six genotypes (one genotype per population). Therefore, we examined variation in gene expression among parental clones in six genotypes and variation among offspring clones in only two genotypes. There was also some mortality within the parents in two of the genotypes (H2 and J3, see Figure 1). Twelve target genes were investigated in the parents. The examined genes were chosen to represent a general physiological response of the earthworms to altered conditions. These included stress-response genes: metallothionein (MT), heat shock protein 40 (HSP40), and heat shock protein 70 (HSP70); as well as genes expected to be involved in metabolism: aldo/keto reductase (AkRed), carbonyl reductase (CarRed), chitinase domain (ChitDo), chymotrypsin inhibitor (ChymInh), dehydrogenase (DeHyd), (similar to) fucosidase (Fuco), leucine aminopeptidase-like protein (Leuc), pyruvate dehydrogenase (Pyr), and L-xylulose reductase (Xyl). We also included analyses of four potential reference genes: 18S-rDNA, 28S-rDNA, peptidylprolyl isomerase (PepIso) and tubulin (Tub). All primers, except those for MT, 18S and 28S [described in 24], and HSP70 [23], were designed based on a draft transcriptome of *D. octaedra* (shared by M. Holmstrup, unpublished, see supporting information S2 Table). A subset of the target genes (AkRed, ChitDo, ChymInh, DeHyd, and MT) were analyzed in the offspring. These were chosen as the most interesting based on the observed variation in gene expression in the parents.

For RNA extraction, we used the Aurum Total RNA mini kit (Bio-Rad) following the manufacturer's protocol including *DNase I* treatment. The concentration of the extracted RNA was measured using the Qubit RNA Assay Kit and Qubit Fluorometer (Invitrogen, Turner BioSystems). For cDNA synthesis, we used the iScript cDNA synthesis kit (Bio-Rad) following the manufacturer's protocol, using 35 ng of RNA per reaction. After synthesis, cDNA was diluted 1:5. Real-time quantitative polymerase chain reactions (qPCR) were performed using IQ SYBR green supermix (Bio-Rad). Prior to analysis, qPCR conditions were optimized and efficiencies were checked with a dilution series (5 points, 10-fold dilutions) (see supporting information S2 Table). In each reaction we used 1 μl of cDNA template and 0.5 μM of each primer in a final reaction vol-

ume of 20 µl (to estimate efficiency) or 10 µl (for the gene expression comparisons). Three replicate reactions for each sample were prepared and an inter-run calibrator was used. For all reactions, we used a CFX96 C1000 Touch Thermal Cycler (Bio-Rad) with the following protocol: 94°C for 2 minutes and then 40 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 15 sec followed by a plate read. A melt curve analysis was done at the end of each amplification reaction, and melt curves were checked to ensure that there was only a single PCR-product.

Data analysis

Raw data and stability of the reference genes was checked with qBase+ (Biogazelle). Data from failed reactions (Cq > 35) and outlying technical replicates (> 1 cycle difference) were removed. Based on our previous study [24], we expected that 18S and 28S would be useful reference genes. However, even though these genes showed stable expression in the parent dataset, their expression was too variable in the offspring dataset, which could be due the age of the earthworms (possibly more variation in the growth stage) or due to variation in sample quality/quantity. Two other potential reference genes were tested (PepIso and Tub), but expression of these genes also varied among the samples (in both parent and offspring datasets). Therefore, we normalized the Cq -values with NORMA-Gene [33], which utilizes the entire dataset of the target genes to reduce systematic and artificial between-replicate bias with a least squares method.

Since our main study question was how much variation in gene expression exists between the groups (within genotypes vs. over all genotypes), we used the normalized Cq-values to calculate estimates of variation within the groups. The estimate of variation (EV) was calculated from the sample variance adjusted with the corresponding degrees of freedom:

$$EV = \frac{\sum \text{variance} * (N_x - 1)}{N_a - G}$$

$\sum$variance= sum of all the sample variances for the group for which EV is calculated
N_x= number of observations in the group for which EV is calculated
N_a= number of all observations
G= number of the groups for which EV is calculated

EV within individuals was calculated from the Cq-values of technical replicates. Similarly, we calculated EV within "families" from the Cq-values of all offspring from a single parent (for the offspring dataset only). EVs within genotypes were calculated from Cq-values of all individuals having the same genotype (for parents and offspring separately). Finally, EV over all genotypes was calculated from Cq-values from all samples, both for the parent dataset and the offspring dataset. All EV- values were calculated for each gene separately.

We then evaluated whether there were differences in the estimates of variation (EV) between the different groups (within individuals, within families,

within genotypes, and over all genotypes). First, these comparisons were made using repeated measures ANOVA with gene as the repeated factor (repeated measures ANOVA was chosen in order to take the dependence in the dataset into account.). There were 12 genes in the parent generation and five genes in the offspring generation; and additionally we compared the same subset of five genes in both datasets (parents and offspring). Pair-wise comparisons were done with LSD post hoc test. Second, comparisons were done for each gene separately using Bartlett's test to see if the variation in EV between the different groups was the same for all genes, and pairwise comparisons were done using the F-test. We also tested whether there were significant differences in the estimate of variation between different genotypes (in parents) and between different families (in the offspring dataset) with repeated measures ANOVA. Although it was not our primary focus, differences between populations (which is also a between genotypes comparison for offspring, since there is one genotype from each population) were tested using the Wilcoxon signed-rank test, and differences between parents and offspring with the Mann-Whitney U test. The signed rank test was chosen as a robust (against non-normality) method for comparison of two related (here, gene-wise) samples.

In addition to variation in gene expression (EV), the levels of gene expression were compared, using relative quantity (RQ) values calculated in qBase+. Relative Quantity values are linear transformations of the Cq-values converted with the efficiencies of the qPCR reactions taken into account [34]. As for the EV-values, these were normalized using NORMA-gene [33]. Normalized RQ-values were used to compare gene expression levels between offspring of the same genotype (but from different parents) i.e. offspring "families", between parents and offspring of the same genotype, between different genotypes (H1, H2, H3, J1, J2 and J3), and between individuals originating from the two different populations (Harjavalta and Jyväskylä). The Mann-Whitney test was used when comparing two groups (two populations or offspring genotype to parent genotype) and the Kruskal-Wallis test was used in cases of more than two groups (different genotypes or families). All statistical analyses were carried out in Excel and in IBM Statistics SPSS 20.

RESULTS

Microsatellite results

Altogether 170 individuals were genotyped. Genotypes were checked from eight single-worm cultures and 12 pair-worm cultures (two earthworms put in each culture and allowed to produce cocoons). Not all parent individuals survived in culture until DNA extraction and there were varying numbers of offspring per culture. Supporting information S3 Table details all individuals and their multi-locus genotypes (allele sizes). Of the five microsatellite loci amplified, one (DO4) was monomorphic. We found only one multi-locus genotype in each of the single-worm cultures: all earthworms from these cultures had the

same genotype, including the parent earthworm when it was available for comparison. One or two multi-locus genotypes were found in each of the pair-worm cultures. Multi-locus genotypes from the offspring in these cultures matched one or the other of the parents (when comparison was possible).

Gene expression variation

We examined variation in the expression of 12 or five target genes (in parents and offspring, respectively) and compared the extent of variation within genotypes and over all genotypes. Combining data from all genes, the estimates of variation (EV) differed significantly between the different groups in the study design (within individuals, within families, within genotypes, and over all genotypes) both in the parent dataset (repeated measures ANOVA, $F = 25.103$, $df_1 = 1.169$, $df_2 = 12.589$, $p < 0.001$; Greenhouse-Geisser correction of the df was used due to non-sphericity, $\varepsilon = 0.584$), and in the offspring dataset ($F = 8.033$, $df_1 = 3$, $df_2 = 12$, $p = 0.003$). Variation increased from within individuals to within families (in offspring) to within genotypes to over all genotypes in both datasets (Figure 2). Pairwise comparisons revealed that in the parent dataset there were significant differences in variation between all groups, whereas in the offspring dataset a significant difference was found only in comparisons to EV within individuals; EV within families, within genotypes and over all genotypes did not differ from each other (Table 2). When analyzing the same subset of genes in the parent data and in the offspring data (five genes), a significant difference was found between the groups ($F = 5.848$, $df_1 = 2$, $df_2 = 8$, $p = 0.027$), but the difference was significant only between within individuals and within genotypes (LSD, mean difference = 0.504, $p = 0.034$) and not between EV within genotypes and EV over all genotypes (LSD, mean difference = 0.736, $p = 0.155$) (Supporting information S4 Figure). Analyzing the genes separately, a significant difference in EV between these groups was found in all genes except AkRed, but pairwise comparisons revealed no significant differences within genotypes and over all genotypes in some genes (ChymInh, Dehyd and HSP40 in parent dataset, all genes except ChymInh in offspring dataset; Supporting information S5 Table).

In the parent dataset, different genotypes did not differ significantly in EV ($F = 0.469$, $df_1 = 2.284$, $df_2 = 25.129$, $p = 0.656$; Greenhouse-Geisser correction of df_2 was used due to non-sphericity, $\varepsilon = 0.457$), and genotypes originating from the uncontaminated site (J) did not differ in EV from the genotypes originating from the contaminated site (H) (Wilcoxon signed-rank test, $Z = -1.02$, $p = 0.308$, Supporting information S6 Figure). The same patterns were observed when analyzing only the subset of five genes from the parent data (data not shown). In the offspring dataset, there was higher EV in the genotype that originated from the uncontaminated site (genotype J1) than the one that originated from the contaminated site (genotype H1)($Z = -2.023$, $p = 0.043$)(Supporting information S7 Figure). There was no significant difference between the EVs of different families ($F = 1.721$, $df_1 = 2.226$, $df_2 = 8.906$, $p = 0.234$; Greenhouse-Geisser correction of df_2 was used due to non-sphericity, $\varepsilon = 0.445$, Supporting information

S8 Figure). However, the variation within genotypes was significantly higher in the offspring than in the parents (Mann-Whitney, U = 9.00, p = 0.027, Figure 2).

Despite the fact that variation in gene expression (EV) was similar in genotypes in the parent dataset, when comparing gene expression levels (RQ), different genotypes showed differences in the expression of most genes both in the parent dataset and in the offspring dataset (Supporting information S9 Table). In these cases the differences were mostly due to one genotype (J3 in the parent dataset, H1 in the offspring dataset) having higher expression than the other genotypes (Supporting information S10 Figure, Figure 3). Expression did not differ between earthworms originating from the two different populations in any of the genes in the parent dataset (Supporting information S11 Table).

A difference in gene expression (RQ) levels among families was observed in one gene out of five. Offspring from different parents (but sharing the same genotype) showed a significant difference in the expression of ChymInh, which was noted in both offspring genotypes, H1 (Kruskal-Wallis, χ^2= 11.06, df = 2, p = 0.004) and J1 (χ^2 = 7.62, df = 2, p = 0.022; Figure 3). Gene expression of offspring differed from that of parents with the same genotype in H1 (all offspring of genotype H1 compared to all parents of genotype H1) in two genes: ChymInh (Mann-Whitney, U = 7.00, df = 1, p = 0.008) and MT (U = 2.00, df =1, p = 0.002). But, when comparing the expression of the offspring to their own parent, expression did not differ significantly (data not shown). Expression did not differ between parents and offspring in genotype J1 in any of the genes.

DISCUSSION

Given that gene expression is noisy [3] and potentially under the influence of epigenetic regulation, assuming that genetically identical individuals exposed to the same conditions will express genes at about the same levels might be unrealistic. We used microsatellite markers to determine whether the earthworm *Dendrobaena octaedra* produces clonal offspring, as expected for an organism with apomictic parthenogenesis, and then we measured gene expression variation both within and among clonal genotypes. Our results show that gene expression can differ between genetically identical individuals exposed to the same conditions and that the degree of variation can depend on clonal genotype and the genes being investigated. This suggests that results of comparative studies of gene expression phenotypes could be affected by variation within genotypes. Some genotypes show more variation than others, so results could be biased by what genotypes happen to be included in the sample or they could be specific to a single genotype only.

One explanation for finding variation in gene expression within a single clonal genotype could be that there is unidentified genetic variation within the sample and that the individuals are not really clones. For example, in our study, there is a possibility that mutations have occurred during the culturing time (1-2 generations) and that the individuals are genetically different despite originat-

ing from a single parent. Nota and colleagues [14] showed that there can be very distinct differences in gene expression between different genotypes of *Folsomia candida* originating from the same clonal line. Similarly, Dudycha and colleagues [9] found differences in gene expression between different clones within *Daphnia* ecotypes. Our genotyping with the microsatellite markers described here confirmed that offspring in our cultures shared the same multi-locus genotype, but this conclusion is necessarily limited by the number of genetic markers used in genotyping. It might be that genetic differences were not surveyed or were overlooked, and our conclusions could be stronger if more genetic markers had been used. Nevertheless, there is strong evidence for apomictic parthenogenesis in this species [25, 26, 27]. And, even though Simonsen and Holmstrup [28] found some differences between offspring and their parent in allozyme markers, most parents and offspring in their study were identical, and the variation they observed could also have derived from variation in gene expression. Therefore, in our study it is likely that individuals from the same culture are truly clones and that the variation in the gene expression we observed is due to plasticity in gene expression.

We observed more variation in gene expression (EV) in the offspring than in the parents in this study, and also offspring expressed some genes differently than the adults of the same genotype. Previous work has shown that gene expression can change drastically during adolescence, e.g. in *Caenorhabditis elegans* [35], and variation in gene expression is also known to increase in aging animals [36, 37]. Difference in age and physiology of the two month old juveniles compared to the mature adults in our experiment could explain the difference in variation in gene expression. Carry-over effects from previous experience of the parents might be one explanation: parents were first raised to adulthood in uncontaminated organic rich soil and then exposed to copper contamination, whereas offspring were only exposed to contaminated soil (after hatching). Another possible reason for more variation in the offspring than in the parents could be that the tissues used in RNA extraction were different: in the parents only a small part from the anterior end was used and in the offspring about half of the earthworm was used for RNA extraction. In the offspring samples there was likely a larger variety of tissue types, which could have led to more variation, since different tissues express genes differently [6, 7].

Nevertheless, in the parent generation we found less variation within genotypes than over all genotypes. Even though there was significant variation in gene expression among individuals sharing the same genotype, genetically identical individuals were more similar in their gene expression than individuals that were genetically different. In the offspring dataset, on the other hand, and when we analyzed only a subset of the genes in the parent dataset, the variation in gene expression within genotypes was as high as the variation over all genotypes. Variation was high even within families (among offspring that were from the same parent). This result could be due to our choice of five genes to be analyzed in the offspring dataset, which were chosen specifically because they showed more variation than other genes in the parental dataset. We might have

found a different result if data for all 12 genes had been collected for the offspring.

Variation in gene expression could be beneficial to organisms when they are exposed to changing conditions, and in evolutionary adaptation in the long run [1, 2, 3, 38]. Genes related to stress have been found to show more variation than other genes that have basic cell maintenance functions [39]. In our study, the three stress related genes, HSP40, HSP70, and MT showed high variation within genotypes, but not necessarily more variation than did many of the genes related to metabolism. One gene, ChymInh, which showed significant gene expression differences within genotypes, is an inhibitor of the digestive enzyme chymotrypsin, but it has also been shown to be involved in an immune response [40]. Thus, it is possible that it is a stress related gene of sorts in this species as well.

In addition to specific genes showing more variation than others, our results also indicate that specific genotypes (e.g J3), might contribute more variation to population gene expression responses than others. Different asexual lineages of *Dendrobaena octaedra* in natural populations might be more or less responsive to environmental conditions. For example, in our previous study [24], we found that *D. octaedra* from populations in either metal-contaminated or uncontaminated soil differed in metallothionein (MT) gene expression based on samples of 41 and 42 individuals. A similar comparison of the few genotypes included in this study showed no difference in expression of MT between the two populations, emphasizing the fact that sufficient samples should be taken in order to make conclusions about population responses. The level of MT gene expression (RQ) of J3 and that of the other genotypes included in this study are within the range of expression levels that we found earlier [24].

Determining the mechanisms behind the gene expression variation among individuals sharing the same genotype is beyond the scope of this study. Some possible explanations can be found from studies on cell-to-cell -variation in gene expression [38]. For example, transcriptional bursts in gene expression, when genes transition between transcriptional activity and inactivity due to chromatin remodeling [38], might be one mechanism. Environmental differences could still play a part: despite mixing the Cu in the experimental soil thoroughly, there could have been slightly different concentrations of Cu in the soil and other small differences in the conditions that might have led to different gene expression responses. In addition, *D. octaedra* are known to avoid patches of soil with highest contamination levels [41]. Quality of the ingested material could also be varying, which could lead to differences in expression of the genes related to digestion. Epigenetic regulation could be another possible explanation for our results. DNA methylation occurs in earthworms in association with metal tolerance [42]. Whether DNA methylation occurs in *D. octaedra* and, if so, whether DNA methylation affects gene expression in this species is not known.

CONCLUSIONS

Studies comparing gene expression phenotypes should take into account the considerable variation in gene expression that can exist even between genetically identical individuals. Variation within genotypes may be significant, as large as that observed among genotypes. Thus, many different genotypes should be used to get a reliable average of the effect (e.g. metal exposure) on gene expression. This is especially important for e.g. ecotoxicological studies, when conclusions are often based on the results from study of a single clonal or inbred line. When using clonal lines, it should be confirmed that individuals truly are genetically identical, and even when they are, gene expression should not be assumed to be identical. More research into what causes differences in gene expression among clones and the practical implications of this variation is needed.

Acknowledgements

The authors are grateful to Annaliisa Kankainen for help in the statistical analyses, and to Martin Chilman, Julie Tessier and Tarja Työläjärvi for assistance in the lab, and to Martin Holmstrup for sharing a draft transcriptome of *D. octaedra.*

REFERENCES

1. Levine, M., Tjian, R. Transcription regulation and animal diversity. Nature 2003; 424:147-151.
2. Lopez-Maury, L., Marquerat, S., Bähler, J. Tuning gene expression to changing environments: from rapid responses to evolutionary adaptation. Nature Reviews Genetics 2008; 9:583-593.
3. Viney, M., Reece, S. Adaptive noise. Proc. R. Soc. B 2013; 280: 20131104
4. Roelofs, D., Aarts, M.G.M., Schat, H., van Straalen, N.M. Functional ecological genomics to demonstrate general and specific responses to abiotic stress. Funct Ecol.2008; 22:8-18.
5. Nadal, E., Ammerer, G., Posas, F. Controlling gene expression in response to stress. Nature Reviews Genetics 2011; 12: 833-845.
6. Rué, P., & Martinez Arias, A. Cell dynamics and gene expression control in tissue homeostasis and development. Mol Syst Biol. 2015; 11(2): 792.
7. Rosa, B., Jasmer, D. Mitreva, M. Genome-Wide Tissue-Specific Gene Expression, Co-expression and Regulation of Co-expressed Genes in Adult Nematode *Ascaris suum.* PLoS Negl Trop Dis.2014; 8 (2): e2678.
8. Dalziel, A., Rogers, S., Schulte, P. Linking genotypes to phenotypes and fitness: how mechanistic biology can inform molecular ecology. Mol Ecol. 2009; 18: 4997–5017.
9. Dudycha, J., Brandon, C., Deitz, K. Population genomics of resource exploitation: insights from gene expression profiles of two Daphnia ecotypes fed alternate resources. Ecol Evol. 2012; 2: 329-340.

10. Wong, A., Gottesman, I., Petronis, A. Phenotypic differences in genetically identical organisms: the epigenetic perspective. Hum Mol Genet. 2005; 14: R11-R18.
11. Ong-Abdullah, M., Ordway, J., Jiang, N., Ooi, S, Kok, S., Sarpan, N., Azimi, N., Hashim, A., Ishak, Z., Rosli, S., Malike, F., Bakar, N., Marjuni, M., Abdullah, N., Yaakub, Z., Amiruddin, M., Nookiah, R., Singh, R., Low, E., Chan, K., Azizi, N., Smith, S., Bacher, B., Budiman, M., Van Brunt, A., Wischmeyer, C., Beil, M., Hogan, M., Lakey, N., Lim, C., Arulandoo, X., Wong, C., Choo, C., Wong, W., Kwan, Y., Alwee, S., Sambanthamurthi, R., Martienssen, R. Loss of *Karma* transposon methylation underlies the mantled somaclonal variant of oil palm. Nature 2015; 24: 525-533.
12. Wolff, G. Variability in Gene Expression and Tumor Formation within Genetically Homogeneous Animal Populations in Bioassays. Fundam Appl Toxicol 1996; 29: 176-184.
13. Pritchard, C., Coil, D., Hawley, S., Hsu, L., Nelson, P. The contributions of normal variation and genetic background to mammalian gene expression. Genome Biology 2006; 7: R26.
14. Nota, B., de Korte, M., Ylstra, B., van Straalen, N.M., Roelofs, D. Genetic variation in parthenogenetic collembolans is associated with differences in fitness and cadmium-induced transcriptome. Environ. Sci. Technol., 2013; 47:1155−1162.
15. Edwards, C.A., Bohlen, P.J. Biology and Ecology of Earthworms, 1996; third ed. Chapman & Hall, London.
16. Terhivuo, J., Saura, A. Dispersal and clonal diversity of North-European parthenogenetic earthworms. Biol. Invasions 2006; 8:1205-1218.
17. Hansen, P. L., Holmstrup, M., Bayley, M., & Simonsen, V. Low genetic variation for *Dendrobaena octaedra* from Greenland compared to populations from Europe and North America: Refuge or selection? Pedobiologia 2006; 50: 225-234
18. Haimi, J., Knott, K.E., Yliniemi, I. Does metal contamination affect clonal diversity of the parthenogenetic earthworm *Dendrobaena octaedra*? Eur J Soil Biol 2007; 43:268-272.
19. Simonsen, V., Laskowski, R., Bayley, M., Holmstrup, M. Low impact of metal pollution on genetic variation in the earthworm *Dendrobaena octaedra* measured by allozymes. Pedobiologia 2008; 52: 51-60.
20. Knott, K.E. & Haimi, J. High mitochondrial DNA sequence diversity in the parthenogenetic earthworm *Dendrobaena octaedra*. Heredity 2010; 105:341–347.
21. Fisker, K.V., Sørensen, J.G., Damgaard, C., Pedersen, K.L., Holmstrup, M. Genetic adaptation of earthworms to copper pollution: is adaptation associated with fitness costs in *Dendrobaena octaedra*? Ecotoxicology 2011; 20:563–573.
22. Fisker, K.V., Sørensen, J.G., Holmstrup, M. No costs on freeze tolerance in genetically copper adapted earthworm populations (*Dendrobaena octaedra*). Comp. Biochem. Physiol. C 2011; 154:204–207.

23. Fisker, K.V., Holmstrup, M., Sørensen, J.G. (2013) Variation in metallothionein gene expression is associated with adaptation to copper in the earthworm *Dendrobaena octaedra*. Comp. Biochem. Physiol. C. 2013; 157:220-226.
24. Mustonen, M., Haimi, J., Väisänen, A., Knott, K.E. Metallothionein gene expression differs in earthworm populations with different exposure history. Ecotoxicology 2014; 23:1732-1743.
25. Omodeo P. Cariologia dei Lumbricidae. II. Contributo. Caryologia 1955; 8:135–178.
26. Hongell, K., Terhivuo, J. Chromosomal status of the parthenogenetic earthworm *Dendrobaena octaedra* (Sav)(Oligochaete, Lumbricidae) in southern Finland. Hereditas 1989; 110(2):179-182.
27. Terhivuo, J., Saura, A. Allozyme variation in parthenogenetic *Dendrobaena octaedra* (Oligochaeta, Lumbricidae) populations of eastern Fennoscandia. Pedobiologia 1990; 34 (2): 113-139.
28. Simonsen, V., Holmstrup, M. Deviation from apomictic reproduction in *Dendrobaena octaedra*? Hereditas 2008; 145: 212-214.
29. Rozen, A. Effect of cadmium on life-history parameters in *Dendrobaena octaedra* (Lumbricidae: Oligochaeta) populations originating from forests differently polluted with heavy metals. Soil Biol Biochem 2006; 38: 489-503.
30. Zane, L., Bargelloni, L., Patarnello, T. Strategies for microsatellite isolation: a review. Mol Ecol. 2002; 11: 1-16.
31. Grapputo, A. Development and characterization of microsatellite markers in the Colorado potato beetle, *Leptinotarsa decemlineata*. Molecular Ecology Notes 2006; 6: 1177–1179.
32. Rozen, S., Skaletsky, H. Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol. 2000; 132:365-86.
33. Heckmann, L., Sorensen, P., Krogh, P., Sorensen, J. NORMA-Gene: A simple and robust method for qPCR normalization based on target gene data. BMC Bioinformatics 2011; 12-250.
34. Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., Vandesompele, J., qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biology 2007; 8: R19.
35. Snoek, B., Sterken, M., Volkers, R., Klatter, M., Bosman, K., Bevers, R., Riksen, J., Smant, G., Cossins, A., Kammenga, J. A rapid and massive gene expression shift marking adolescent transition in *C. elegans*. Scientific Reports 2014; 4: 3912.
36. Bahar, R., Hartmann, C., Rodriguez, K., Denny, A., Busuttil, R., Dolle, M., Calder, B., Chisholm, G., Pollock, B., Klein, C., Vijg, J. Increased cell-to-cell variation in gene expression in ageing mouse heart. Nature 2006; 441: 1011-1014.
37. Vinuela, A., Snoek, B., Riksen, J., Kammenga, J. Genome-wide gene expression regulation as a function of genotype and age in *C. elegans*. Genome Res. 2010; 20:929-937.

38. Raj, A., van Oudenaarden, A. Nature, nurture, or change: Stochastic Gene Expression and Its Consequences. Cell 2008; 135:216-226.
39. Blake, W., Balazsi, G., Kohanski, M., Isaacs, F., Murphy, K., Kuang, Y., Cantor, C., Walt, D., Collins, J. Phenotypic Consequences of Promoter-Mediated Transcriptional Noise. Mol Cell 2006; 24: 853-865.
40. Schoofs, L., Clynen, E., Salzet, M. Trypsin and chymotrypsin inhibitors in insects and gut leeches. Curr Pharm Des. 2002; 8(7): 483-91.
41. Lukkari, T. & Haimi, J. Avoidance of Cu- and Zn-contaminated soil by three ecologically different earthworm species. Ecotox. Environ. Safe., 2005; 62:35-41.
42. Kille, P., Andre, J., Anderson, C., Ang, H., Bruford, M., Bundy, J., Donnelly, R., Hodson, M., Juma, G., Lahive, E., Morgan, A., Sturzenbaum, S., Spurgeon, D. DNA sequence variation and methylation in an arsenic tolerant earthworm population. Soil Biol Biochem 2013; 57: 524-532.

Table 1. Microsatellite loci in *Dendrobaena octaedra.*

Locus name	Repeat	Sequence of F-primer (5′-3′)	Sequence of R-primer (5′-3′)	Expected product size (bp)	Fluorescent ABI dyes
DO1	(GAAGA-GA_3/GA_{11} /$GAAA_4$) interrupted	GAGTCAGTTG-GACAATTACA CTGG	TCTTCCTCTCTTAT ACATGTAAGTCAA	234	Red (PET)
DO2	$(GA)_{10}$	ACATGCTGCTTG GTTCCTTC	AAGCCGATGCA-CAGGAAAG	160	Blue (6-FAM)
DO3	$(GACA)_{10}$	ATGCGGATTA TGGAGACCAA	TGCAGCAGTC TTGCTCTTTC	183	Green (VIC)
DO4	$(GA)_9$	GCCTTAGCGA CCGTATTTTG	TCGCATAGC TGTTGTTGCAT	229	Yellow/ black (NED)
DO6	$(GA)_6$ $(N)_{16}$ $(GA)_4$	GTGGTCCTGTTT ACTCCCAAG	TCCATCTCTC-CATGTTTCCTC	100	Red (PET)

Table 2. Differences in the estimate of variation values (EV) between each group in the study design in both in the parent (12 genes) and offspring datasets (five genes). Pair-wise comparisons for differences in EV were performed using LSD post hoc test. Note that "within family" comparisons are not possible in the parent dataset.

		Within families		Within genotypes	
		Mean differen-ce	p	Mean diffe-rence	p
Parents	Within individuals	-	-	0.370	0.001
	Within genotypes	-	-	-	-
	Over all genotypes	-	-	0.995	0.002
Offs-pring	Within individuals	0.913	0.028	1.190	0.034
	Within families	-	-	0.227	0.181
	Within genotypes	-	-	-	-
	Over all genotypes	0.122	0.350	0.399	0.221

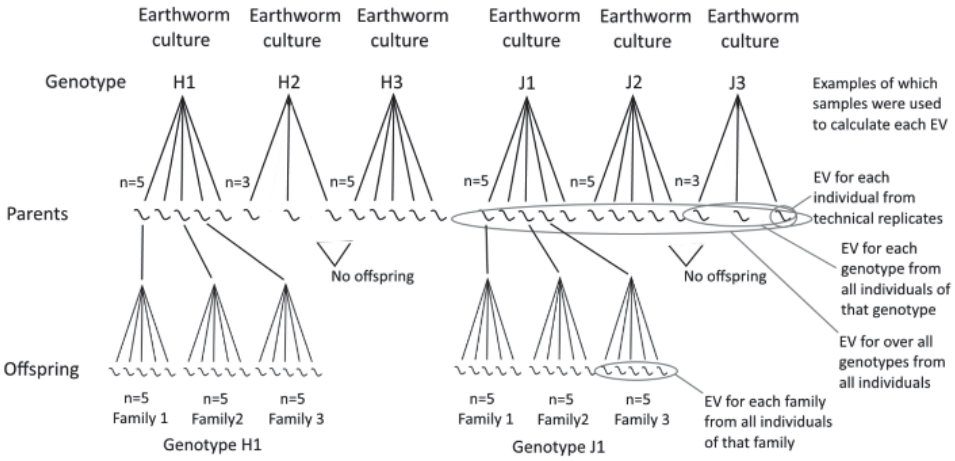


Figure 1. Figure showing the experimental setup and which genotypes were used in the analyses from the parent and offspring generations, (in the parent generation, five individuals per genotype were used, except for genotypes H2 and J3, from which there were only three individuals per genotype). Also shown are examples of which samples were used to calculate the different estimates of variation (EV), see section 2.4.1 in Materials and Methods.

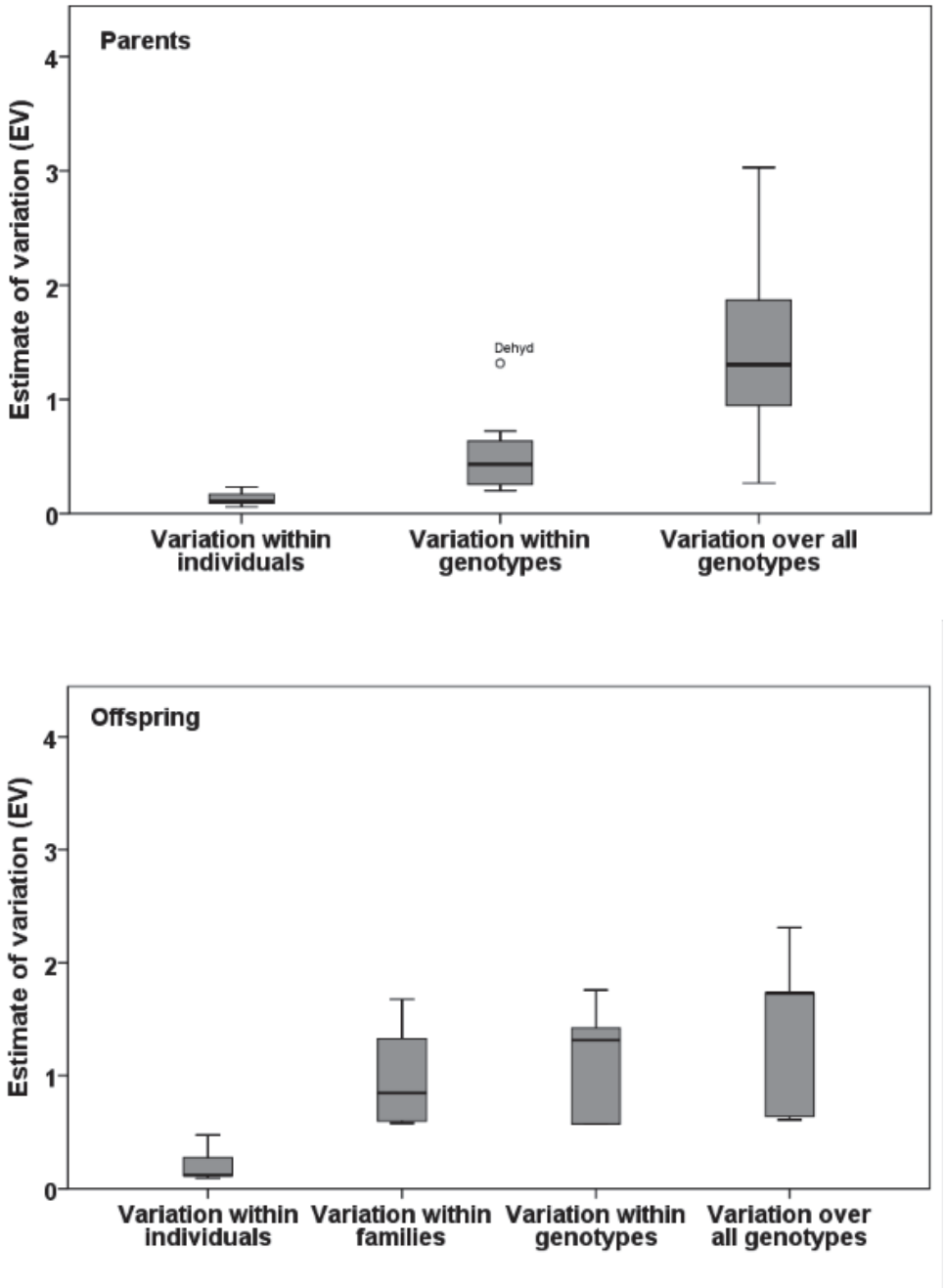


Figure 2. Estimate of variation (EV) in the different groups (within individuals, within families, within genotypes and over all genotypes).

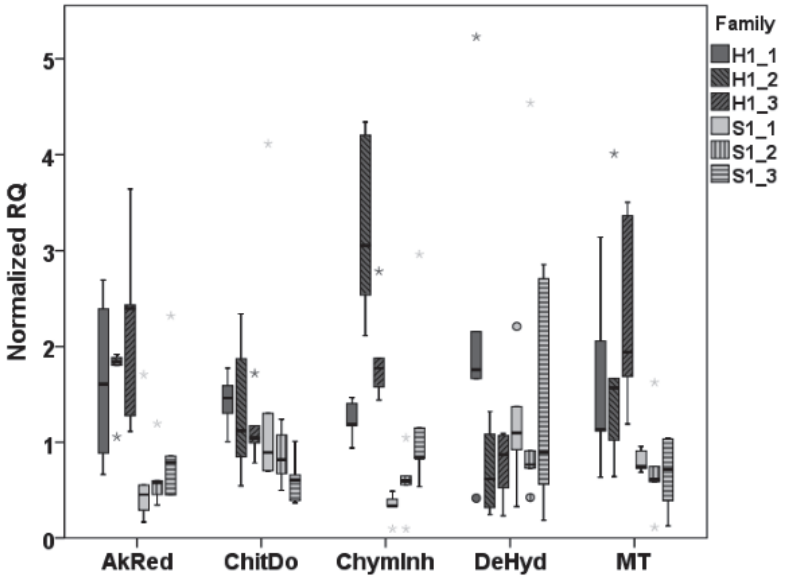


Figure 3. Gene expression (RQ) in offspring of each family.

SUPPLEMENTARY DATA

S1 Table. Allele sizes of loci DO1, DO2, DO3, DO4 and DO6 from earthworms from each of the cultures used in the gene expression experiment.

Culture	DO1	DO2	DO3	DO4	DO6
H1	231/237	140/153	165/173/177	211/213	96/100
H2	231	140	173/181/189	211/213	96
H3	229/231/233/241/245/253	140/147/151	177/185/187/193	211/213	90/96
J1	228/234/238/240/244	140/153	173/177/193	211/213	90/96
J2	228/234/236/238/240	140/147/151	177/185/187/192	211/213	90/96
J3	228/240	140/157	177	211/213	96/98

S2 Table. Primer sequences used in qPCR and reaction efficiencies.

Gene	Primer (5′-3′)	Efficiency %
MT-2	F: ACACTCAGTGCTGTGGCAGCG	103,5
	R: GGCTGCGCACTTGCAGGC	
HSP40	F: ATGTCGGCTGGGATGTTGTT	99,8
	R: AAGAAAGGATGGAAGCCGGG	
HSP70	F: AGCTGAGCATCGAGGAGAAG	105,6
	R: TCCTTGAAGTCCTCCACGTC	
AkRed	F: GCTGATCGAGGCAGATCCTT	96,2
	R: CCAAGTCCACGAACCCTGAA	
CarRed	F: ATGGCAAGGATTCAGCAGGG	97,1
	R: TGGGGTAGAAACTCCCTCCG	
ChitDo	F: GAGAGCCAGTCGTGTTTGGA	94,9
	R: GAAACTGCAATGGGACGCAG	
ChymInh	F: TCAGCTACCCGGTTCTAGGT	94,8
	R: AAGAGGCCAAAGCACAGGTT	
DeHyd	F: CTGGAAGAGGAAGACCTGGAT	100,9
	R: GACGCTTACGAACACGCTCT	
Fuco	F: ACGCAAGACCACTTCCTACG	95,35
	R: CCCAGGACGGAAATTCTGCT	
Leuc	F: AGCCACAGGAGTCTTCACCAA	99,4
	R: TGCGCCACCATATCTTCCTG	
Pyr	F: CTGGAGCAGTCAGGCATCAA	97,1
	R: GCCTTGCTTTCCATGATCCG	
Xyl	F: ACATGGCTTTGGAGCTTGGA	109,8
	R: ACGGTCATGCCACCATCAAT	
PepIso	F: CGAAGATGCTGGTTCCTCCT	90,6
	R: CACCGCGTCGTTAGGGATTT	
Tub	F: GCCTCGACAACTTCCGATTC	98,9
	R: ATGGAATTCACCGAGGCTGA	
18S	F: ACCACATCCAAGGAAGGCAG	93,3
	R: CCCGAGATCCAACTACGAGC	
28S	F: TGGTGGAGGTCCGCAGCGAT	90,5
	R: CGTTTCGTCCCCAAGGCCTC	

S3 Table. Allele sizes of microsatellite loci DO1, DO2, DO3, DO4 and DO6 for earthworm-families originating either from Harjavalta (H) or Jyväskylä (J). Each earthworm-culture had originally either one (P1) or two parent-earthworms (P1 and P2) which produced offspring (F1). DO4 was monomorphic and not used in genotyping some samples, and DO1 had low signal and in some samples it could not be scored (marked with - in the table). Parent-individual was not always available for genotyping because of high mortality (marked "Not available" in the table).

Single earthworm cultures					
Individual	DO1	DO2	DO3	DO4	DO6
H2P1	231	140	173/181/187	211/213	96
H2F1.1	231	140	173/181/187	211/213	96
H2F1.2	231	140	173/181/187	211/213	96
H2F1.3	231	140	173/181/187	211/213	96
H2F1.4	231	140	173/181/187	211/213	96
H4P1	Not available				
H4F1.2	231	140	173/181/187	211/213	96
H4F1.3	231	140	173/181/187	211/213	96
H4F1.4	231	140	173/181/187	211/213	96
H4F1.5	231	140	173/181/187	211/213	96
H4F1.6	231	140	173/181/187	211/213	96
H4F1.7	231	140	173/181/187	211/213	96
H5P1	217/225/233/241	138/140	165/177/185/193	211/213	96/110
H5F1.1	217/225/233/241	138/140	165/177/185/193	211/213	96/110
H5F1.2	217/225/233/241	138/140	165/177/185/193	211/213	96/110
H5F1.3	217/225/233/241	138/140	165/177/185/193	211/213	96/110
H5F1.4	217/225/233/241	138/140	165/177/185/193	211/213	96/110
H5F1.5	217/225/233/241	138/140	165/177/185/193	211/213	96/110
H5F1.6	217/225/233/241	138/140	165/177/185/193	211/213	96/110
H5F1.7	217/225/233/241	138/140	165/177/185/193	211/213	96/110
H5F1.8	217/225/233/241	138/140	165/177/185/193	211/213	96/110
H5F1.9	217/225/233/241	138/140	165/177/185/193	211/213	96/110
H5F1.10	217/225/233/241	138/140	165/177/185/193	211/213	96/110
H5F1.11	217/225/233/241	138/140	165/177/185/193	211/213	96/110
H5F1.12	217/225/233/241	138/140	165/177/185/193	211/213	96/110
H9P1	219/229/239/248	140/151/173	165/173/177/ 187/190/193	211/213	96
H9F1.1	219/229/239/248	140/151/173	165/173/177/ 187/190/193	211/213	96
H9F1.2	219/229/239/248	140/151/173	165/173/177/ 187/190/193	211/213	96
H9F1.3	219/229/239/248	140/151/173	165/173/177/ 187/190/193	211/213	96
H12P1	231	140	173/181/187	211/213	96
H12F1.1	231	140	173/181/187	211/213	96
H12F1.2	231	140	173/181/187	211/213	96
H12F1.3	231	140	173/181/187	211/213	96
H12F1.4	231	140	173/181/187	211/213	96
H12F1.5	231	140	173/181/187	211/213	96
H12F1.6	231	140	173/181/187	211/213	96
H13P1	Not available				
H13F1.1	231/235	140/147	165/173/177	211/213	96/100
H13F1.2	231/235	140/147	165/173/177	211/213	96/100
H13F1.3	231/235	140/147	165/173/177	211/213	96/100
H13F1.4	231/235	140/147	165/173/177	211/213	96/100
H14P1	231	140	173/181/187	211/213	96
H14F1.1	231	140	173/181/187	211/213	96
H14F1.2	231	140	173/181/187	211/213	96

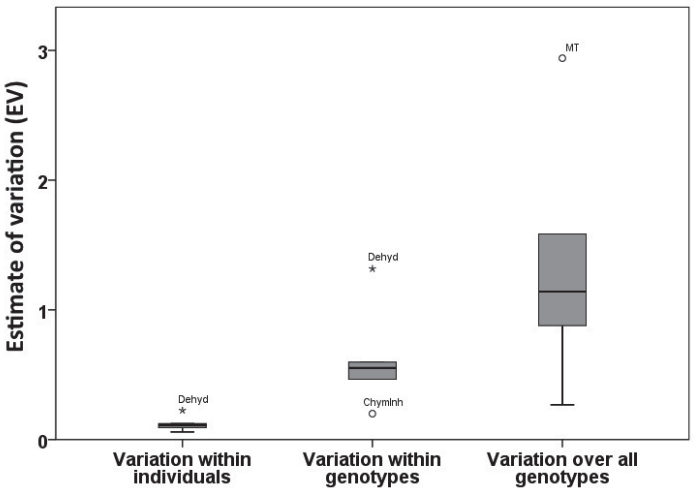
H14F1.3	231	140	173/181/187	211/213	96
H14F1.4	231	140	173/181/187	211/213	96
H14F1.5	231	140	173/181/187	211/213	96
H14F1.6	231	140	173/181/187	211/213	96
H14F1.7	231	140	173/181/187	211/213	96
H15P1	221/227/231/235	138/140/171	165/173/177/185	211/213	96/100
H15F1.1	221/227/231/235	138/140/171	165/173/177/185	211/213	96/100
H15F1.2	221/227/231/235	138/140/171	165/173/177/185	211/213	96/100
H15F1.3	221/227/231/235	138/140/171	165/173/177/185	211/213	96/100
H15F1.4	221/227/231/235	138/140/171	165/173/177/185	211/213	96/100
H15F1.5	221/227/231/235	138/140/171	165/173/177/185	211/213	96/100
H15F1.6	221/227/231/235	138/140/171	165/173/177/185	211/213	96/100
H15F1.7	221/227/231/235	138/140/171	165/173/177/185	211/213	96/100
H15F1.8	221/227/231/235	138/140/171	165/173/177/185	211/213	96/100
H15F1.9	221/227/231/235	138/140/171	165/173/177/185	211/213	96/100
H15F1.10	221/227/231/235	138/140/171	165/173/177/185	211/213	96/100
H15F1.11	221/227/231/235	-	165/173/177/185	211/213	96/100
H15F1.12	221/227/231/235	138/140/171	165/173/177/185	211/213	96/100
H15F1.13	221/227/231/235	138/140/171	165/173/177/185	211/213	96/100
H15F1.14	221/227/231/235	138/140/171	165/173/177/185	211/213	96/100
H15F1.15	221/227/231/235	138/140/171	165/173/177/185	211/213	96/100

Pair earthworm cultures

Individual	DO1	DO2	DO3	DO4	DO6
H112P1	217/225/231/233	138/140	165/177/185/193	211/213	96/100
H112P2	Not available				
H112F1.1	231/233/235	140/147	165/173/177	211/213	96/100
H114P1	Not available				
H114P2	Not available				
H114F1.1	231	140	173/181/187	211/213	96
H114F1.2	231	140	173/181/187	211/213	96
H114F1.3	231	140	173/181/187	211/213	96
H114F1.4	231	140	173/181/187	211/213	96
H114F1.5	231	140	173/181/187	211/213	96
H116P1	231	140	173/181/187	211/213	96
H116P2	231	140	173/181/187	211/213	96
H116F1.1	231	140	173/181/187	211/213	96
H116F1.2	231	140	173/181/187	211/213	96
H118P1	231	140	173/181/187	211/213	96
H118P2	Not available				
H4F1.2	231	140	173/181/187	211/213	96
H4F1.3	231	140	173/181/187	211/213	96
H4F1.4	231	140	173/181/187	211/213	96
H4F1.5	231	140	173/181/187	211/213	96
H4F1.6	231	140	173/181/187	211/213	96
H4F1.7	231	140	173/181/187	211/213	96
H5P1	217/225/233/241	138/140	165/177/185/193	211/213	96/110
H5F1.1	217/225/233/241	138/140	165/177/185/193	211/213	96/110
H5F1.2	217/225/233/241	138/140	165/177/185/193	211/213	96/110
H5F1.3	217/225/233/241	138/140	165/177/185/193	211/213	96/110
H5F1.4	217/225/233/241	138/140	165/177/185/193	211/213	96/110
H5F1.5	217/225/233/241	138/140	165/177/185/193	211/213	96/110
H5F1.6	217/225/233/241	138/140	165/177/185/193	211/213	96/110
H5F1.7	217/225/233/241	138/140	165/177/185/193	211/213	96/110
H5F1.8	217/225/233/241	138/140	165/177/185/193	211/213	96/110
H5F1.9	217/225/233/241	138/140	165/177/185/193	211/213	96/110
H5F1.10	217/225/233/241	138/140	165/177/185/193	211/213	96/110
H5F1.11	217/225/233/241	138/140	165/177/185/193	211/213	96/110
H5F1.12	217/225/233/241	138/140	165/177/185/193	211/213	96/110
H9P1	219/229/239/248	140/151/173	165/173/177/	211/213	96

			187/190/193		
H9F1.1	219/229/239/248	140/151/173	165/173/177/ 187/190/193	211/213	96
H9F1.2	219/229/239/248	140/151/173	165/173/177/ 187/190/193	211/213	96
H9F1.3	219/229/239/248	140/151/173	165/173/177/ 187/190/193	211/213	96
H12P1	231	140	173/181/187	211/213	96
H201F1.6	226/228/230/232/ 234/236/238/246	140	173/181/187	-	96
H201F1.7	226/228/230/232/ 234/236/238/246	140	173/181/187	-	96
H201F1.8	226/228/230/232/ 234/236/238/246	140	173/181/187	-	96
H201F1.9	226/228/230/232/ 234/236/238/246	140	173/181/187	-	96
H201 F1.10	226/228/230/232 /234/236/238/246	140	173/181/187	-	96
H201 F1.11	226/228/230/232 /234/236/238/246	140	173/181/187	-	96
H201 F1.12	226/228/230/232 /234/236/238/246	140	173/181/187	-	96
H201 F1.13	226/228/230/232 /234/236/238/246	140	173/181/187	-	96
H201 F1.14	226/228/230/232/ 234/236/238/246	140	173/181/187	-	96
H201 F1.15	226/228/230/232/ 234/236/238/246	140	173/181/187	-	96
H201 F1.16	226/228/230/232/ 234/236/238/246	140	173/181/187	-	96
H201 F1.17	226/228/230/232/ 234/236/238/246	140	173/181/187	-	96
H201 F1.18	226/228/230/232/ 234/236/238/246	140	173/181/187	-	96
H202P1	-	140/147	165/173/177	-	96
H202P2	Not available				
H202F1.1	226/228/230/232/ 234/236/238/246	140/151	173/181/189	-	96
H202F1.2	-	140/147	165/173/177	-	96
H202F1.3	226/228/230/232/ 234/236/238/246	140/151	173/181/189	-	96
H202F1.4	226/228/230/232/ 234/236/238/246	140/151	173/181/189	-	96
H202F1.5	226/228/230/232/ 234/236/238/246	140/151	173/181/189	-	96
H202F1.6	226/228/230/232/ 234/236/238/246	140/151	173/181/189	-	96
H202F1.7	226/228/230/232/ 234/236/238/246	140/151	173/181/189	-	96
H202F1.8	226/228/230/232/ 234/236/238/246	140/151	173/181/189	-	96
H202F1.9	226/228/230/232/ 234/236/238/246	140/151	173/181/189	-	96
H202 F1.10	226/228/230/232/ 234/236/238/246	140/151	173/181/189	-	96
H202 F1.11	226/228/230/232/ 234/236/238/246	140/151	173/181/189	-	96
H202 F1.12	226/228/230/232/ 234/236/238/246	140/151	173/181/189	-	96

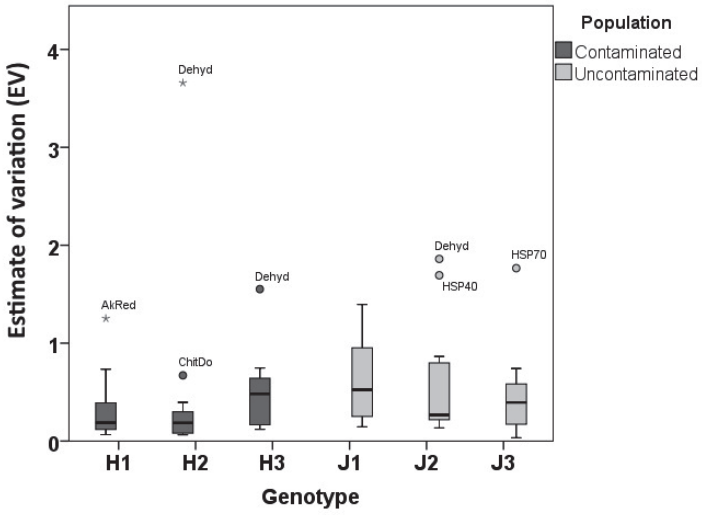
H202P1	-	140/147	165/173/177	-	96
H202P2	Not available				
J122P1	Not available				
J122P2	Not available				
J122F1.1	-	140/151	165/173/177/ 181/196/200	211/213	96/100
J122F1.2	217	140/151	165/173/177/ 181/196/200	211/213	96/100
J122F1.3	217	140/151	165/173/177/ 181/196/200	211/213	96/100
J124P1	232/234/236	138/140/151/157	165/173/181/ 185/190/196	211/213	96
J124P2	Not available				
J124F1.1	232/234/236	138/140/151/157	165/173/181/ 185/190/196	211/213	96
J124F1.2	232/234/236	138/140/151/157	165/173/181/ 185/190/196	211/213	96
J124F1.3	232/234/236	138/140/151/157	165/173/181/ 185/190/196	211/213	96
J124F1.4	232/234/236	138/140/151/157	165/173/181/ 185/190/196	211/213	96
J124F1.5	-	140	173/177/194	211/213	96
J124F1.6	232/234/236	138/140/151/157	165/173/181/ 185/190/196	211/213	96
J124F1.7	232/234/236	138/140/151/157	165/173/181/ 185/190/196	211/213	96
J124F1.8	217	140	173/177/194	211/213	96
J124F1.9	232/234/236	138/140/151/157	165/173/181/ 185/190/196	211/213	96
J130P1	229/231/233/237	140/153	173/177/190/194	211/213	96
J130P2	Not available				
J130F1.1	-	140/153	173/177/190/194	211/213	96
J130F1.2	230/232/234/238	140/153	173/177/190/194	211/213	96
J130F1.3	230/232/234/238	140/153	173/177/190/194	211/213	96
J130F1.4	230/232/234/238	140/153	173/177/190/194	211/213	96
J130F1.5	230/232/234/238	140/153	173/177/190/194	211/213	96
J130F1.6	230/232/234/238	140/153	173/177/190/194	211/213	96
J130F1.7	230/232/234/238	140/153	173/177/190/194	211/213	96
J130F1.8	230/232/234/238	140/153	173/177/190/194	211/213	96
J130F1.9	230/232/234/238	140/153	173/177/190/194	211/213	96
J130F1.10	230/232/234/238	140/153	173/177/190/194	211/213	96
J130F1.11	230/232/234/238	140/153	173/177/190/194	211/213	96
J130F1.12	229/231/233/237	140/153	173/177/190/194	211/213	96
J202P1	Not available				
J202P2	Not available				
J202F1.1	217/227/229/ 231/235/247	140/151	177/194	-	96
J202F1.2	217/227/229/ 231/235/247	140/151	177/194	-	96



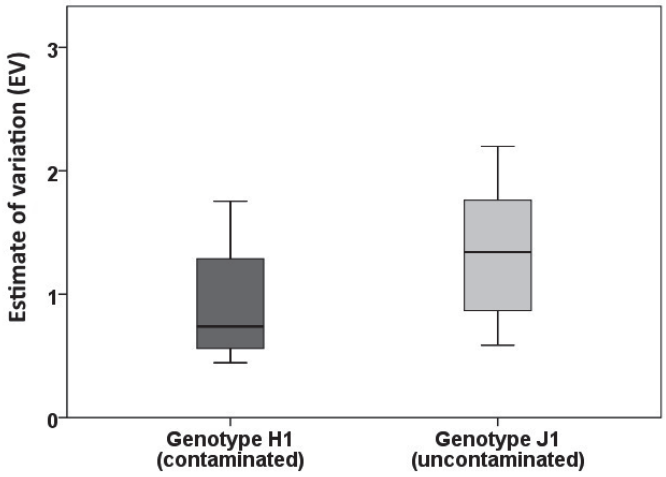
S4 Figure. Estimate of variation (EV) in the different groups (within individuals, within families, within genotypes and over all genotypes) for the parent data when using only the subset of five genes (AkRed, ChitDo, ChymInh, DeHyd and MT).

S5 Table . Difference in the estimate of variation (EV) between different levels (within individuals, within families in offspring-data, within genotypes and over all genotypes) for each gene separately, and pairwise comparisons between the levels: 1. comparison is between within individuals and within families, 2. comparison in between within families and within genotypes for offspring data and within individuals and within genotypes for parent data, and 3. comparison is between within genotypes and over all genotypes. Bartlett test was used for overall analysis and F-test for pairwise comparisons.

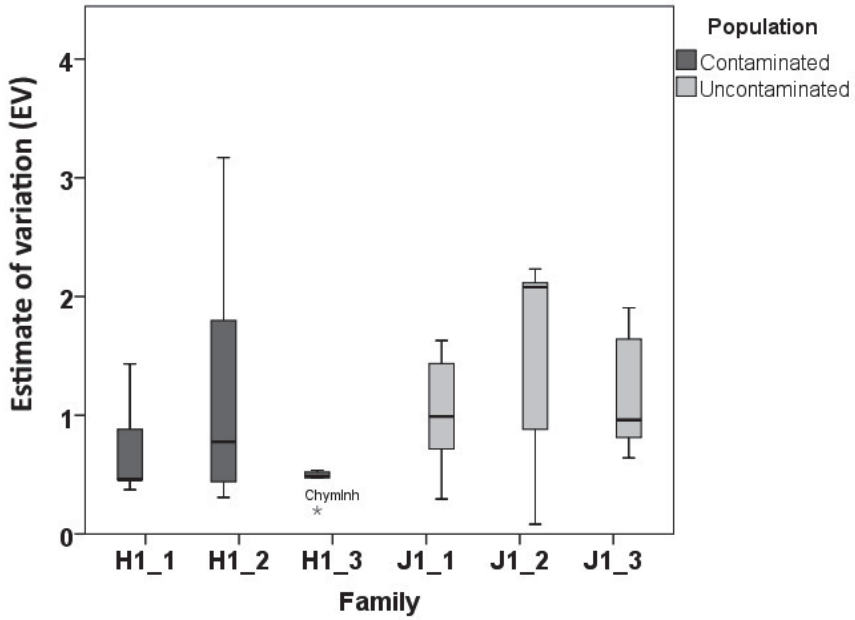
			Overall		1.comparison		2.comparison		3.comparison	
		df	χ^2	P	F	P	F	P	F	P
Parents	AkRed	2	75.56	<0.001	-	-	5.29	<0.001	1.91	0.005
	CarRed	2	129.68	<0.001	-	-	1.84	0.021	7.87	<0.001
	ChitDo	2	60.95	<0.001	-	-	3.76	<0.001	1.89	0.004
	ChymInh	2	38.87	<0.001	-	-	3.36	<0.001	1.34	0.106
	Dehyd	2	42.91	<0.001	-	-	5.85	<0.001	1.20	0.258
	Fuco	2	156.95	<0.001	-	-	5.10	<0.001	4.50	<0.001
	Leuc	2	137.10	<0.001	-	-	2.29	0.001	6.14	<0.001
	Pyr	2	99.97	<0.001	-	-	1.95	0.006	4.81	<0.001
	Xyl	2	98.84	<0.001	-	-	3.08	0.001	4.19	<0.001
	HSP40	2	28.88	<0.001	-	-	3.46	<0.001	1.10	0.351
	HSP70	2	102.89	<0.001	-	-	4.32	<0.001	2.88	<0.001
	MT	2	181.24	<0.001	-	-	5.94	<0.001	5.32	<0.001
OffSpring	AkRed	3	1.84	0.607	1.26	0.268	1.05	0.436	1.12	0.347
	ChitDo	3	46.73	<0.001	4.90	<0.001	1.01	0.482	1.07	0.395
	ChymInh	3	141.26	<0.001	7.44	<0.001	1.68	0.012	1.62	0.016
	Dehyd	3	56.10	<0.001	6.12	<0.001	1.05	0.423	1.01	0.475
	MT	3	133.95	<0.001	14.12	<0.001	1.01	0.479	1.32	0.110



S6 Figure. Estimate of variation in all genes (AkRed, CarRed, ChitDo, ChymInh, DeHyd, Fuco, Leuc, Pyr, Xyl, HSP40, HSP70 and MT) within parent-genotypes (H1,H2, H3, J1, J2 and J3).



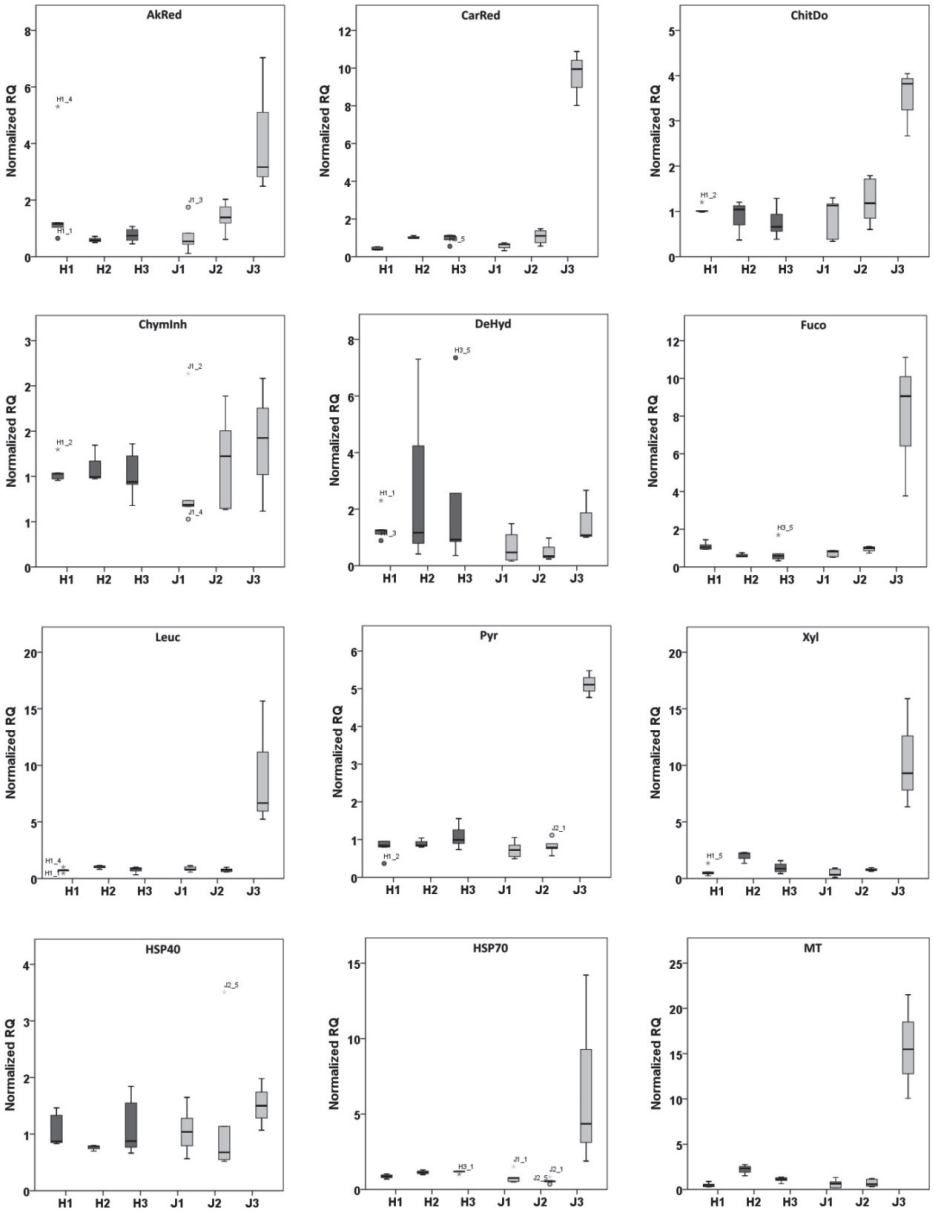
S7 Figure. Estimate of variation (EV) for genotypes H1 (originating from the contaminated site) and J1 (originating from the uncontaminated site) in the offspring dataset.



S8 Figure. Estimate of variation in genes AkRed, ChitDo, ChymInh, DeHyd, Fuco and MT within offspring-families.

S9 Table. Difference in gene expression of each gene between different genotypes. Kruskal Wallis analysis test for the parents and Mann-Whitney test for the offspring.

		df	χ^2	P
Parents	AkRed	5	14.187	0.014
	CarRed	5	18.121	0.003
	ChitDo	5	9.474	0.092
	ChymInh	5	2.249	0.814
	Dehyd	5	6.792	0.237
	Fuco	5	15.519	0.008
	Leuc	5	11.646	0.040
	Pyr	5	10.924	0.053
	Xyl	5	14.259	0.014
	HSP40	5	5.351	0.375
	HSP70	5	17.672	0.003
	MT	5	16.966	0.005
OffSpring	AkRed	1	22.00	<0.001
	ChitDo	1	52.00	0.012
	ChymInh	1	14.00	<0.001
	Dehyd	1	97.00	0.727
	MT	1	27.00	<0.001



S10 Figure. Gene expression (RQ) of each gene in each parent-genotype.

S11 Table. Difference in gene expression of each gene between different populations. Mann-Whitney test.

Gene	U	P
AkRed	60.00	0.209
CarRed	57.00	0.253
ChitDo	53.00	0.106
ChymInh	76.00	0.663
Dehyd	37.00	0.082
Fuco	62.00	0.249
Leuc	67.00	0.369
Pyr	81.00	0.858
Xyl	65.00	0.951
HSP40	79.00	0.778
HSP70	56.00	0.144
MT	80.00	0.817

III

GENETIC DIVERSITY OF AN EARTHWORM AFFECTS ECOSYSTEM FUNCTIONING

by

Marina Mustonen, Jari Haimi & K. Emily Knott

Submitted manuscript

GENETIC DIVERSITY OF AN EARTHWORM AFFECTS ECOSYSTEM FUNCTIONING

Marina Mustonen[1], Jari Haimi[13]

&

K. Emily Knott[1]

[1]Department of Biological and Environmental Science, P.O. Box 35, FI-40014, University of Jyvaskyla, Finland

ABSTRACT

Background: Ecosystem functioning is affected by biodiversity, often through complementary functions or because high-functioning species are more likely to emerge from a species-rich community. Genetic diversity is one level of biodiversity, and a positive effect of higher genetic diversity on different features of ecosystem functioning have also been found. We studied whether genetic diversity of a key decomposer species had an effect on decomposition processes, an important part of terrestrial ecosystem functioning. In a microcosm experiment, we exposed low genetic diversity and high genetic diversity assemblages of the earthworm *Dendrobaena octaedra* to different conditions: uncontaminated soil or soil slightly (100 mg kg^{-1} dry mass) contaminated with Cu; either few (no intentionally added other decomposers) or diverse community (added another earthworm species, enchytraeds and microarthropods) of other soil animals present with *D. octaedra*. We studied the soil decomposition activity and wellbeing of the earthworms through measurements of nitrogen mineralization, CO_2-production, biomass of the earthworms, and their cocoon production, mortality and gene expression.

Results: There were differences between low and high diversity treatments in many parameters, either overall or in specific conditions, e.g. in mineral nitrogen concentration, earthworm growth and in expression of chymotrypsin inhibitor gene, indicating that earthworms in the high diversity treatment had thrived and contributed more in decomposition, especially in contaminated conditions. However, the direction and magnitude of the difference depended on what genotype was present in the low diversity treatment.

Conclusions: Our results suggest that higher genetic diversity of a key species could be beneficial for decomposition activity, especially in contaminated conditions. However, it is not only genetic diversity *per se*, but well-adapted genotypes of the key decomposer species that affects ecosystem functioning.

BACKGROUND

There is often a positive relationship between biodiversity and ecosystem functioning, the processes affecting the flow of energy and cycling of nutrients, e.g. primary production and decomposition. When there are many different species, they can occupy complementary ecological niches and enhance resource use, and high-functioning species are more likely to exist when there is high biodiversity [1, 2, 3, 4, 5]. It is well-known that ecological processes affect evolutionary change, and likewise, recent research has demonstrated that evolutionary change also can take place in timescales that are relevant ecologically, therefore affecting ecological dynamics [6, 7, 8]. Research on the two-way relationship between ecological and evolutionary processes has shown that ecological-evolutionary feedbacks are important for the diversity-functioning relationship [6, 7]. Specifically, this work has demonstrated how evolutionary diversification of organisms can affect ecosystem functioning [6, 8, 9].

Genetic diversity is an important component of biodiversity, and it can have important effects on ecological processes e.g. through increased primary production, faster recovery from disturbances, effects on interspecific competition and effects on decomposition through increased litter diversity [10]. In some cases the effects of genetic diversity can be as important as effects of species diversity, and there are indications that genetic diversity is especially important in highly variable environments or when the environment is subjected to anthropogenic changes [10]. The review by Hughes and colleagues [10] mostly concerns studies with plants, as do other studies that specifically address the effect of genetic diversity on ecosystem functioning [11, 12, 13, 14, 15]. In a meta-analysis, Whitlock [16] found positive correlation between intraspecific diversity and ecological functioning in plants, but the relationship was dependent on whether the focus was on adaptive genetic diversity (positive correlation) or neutral genetic diversity (positive correlation only under a limited set of demographic conditions). Wymore and colleagues [17] also emphasized the importance of the effects of genetic diversity on ecosystem functioning in their review of studies on plants, and they suggested that the same principles could be applied to a multitude of situations and trophic levels, including fauna. Although the effect of faunal genetic diversity on ecosystem processes is less studied, some examples can be found, indicating that higher genetic diversity has a positive effect on some functional parameters e.g. in the marine invertebrate *Bugula neritina* [18] and in the black surfperch *Embiotica jacksoni* [19].

In soil, biodiversity affects ecosystem functioning, but the relative importance of genetic, species and functional diversity explaining the diversity-functioning relationship is still unclear [20, 21, 22]. Some studies suggest that e.g. plant productivity and decomposition are insensitive to loss faunal diversity, and that it is the presence of key-species and functionally different groups, rather than species diversity *per se*, that is important for functional parameters in soils [23, 24, 25, 26], or that the effect of species diversity on ecosystem functioning is inconsistent [27]. On the other hand, other studies have found that

species diversity, in addition to functional differences and the presence of key-species, affects ecosystem functioning [28, 29, 30, 31, 32, 33].

Anthropogenic contaminants can have negative impacts on organisms and reduce species diversity [34, 35, 36, 37]. But, whether or not anthropogenic contaminants impact ecosystem functioning is not always clear [38]. In their systematic review, Johnston and colleagues [38] found that effects of chemical contaminants on ecosystem functioning were negative in up to 70 % of the studies. However, in some studies on the impact of anthropogenic contaminants, the functional measures have not always been identified as such and the results are not interpreted in the context of ecosystem functioning [38]. Fariña and colleagues [39] and Li and colleagues [40] found a positive biodiversity-productivity relationship in metal-contaminated aquatic environments. In soils, metals have immediate biological effects but also long-term effects since they accumulate in the litter layer and soil and are only slowly eliminated [41, 42, 43]. Metals often have negative impact on organisms e.g. through the production of reactive radicals that induce genotoxic damage [44] and so, metal contamination can decrease species diversity [45]. The consequences of a reduction in species diversity in soils are important to study, since soil invertebrates participate in many physical, chemical and biological processes and thus are important for ecosystem functioning [46]. Among soil invertebrates, earthworms are key decomposers in many ecosystems, contributing considerably to nutrient mineralization and soil structure [31, 46, 47, 48, 49] and metal contamination is known to have a negative impact on the species diversity of earthworms [50, 51, 52].

Although species diversity often is diminished due to metal contamination, the same is not necessarily true for the genetic diversity of the species that persist. There are some studies that show negative impact of anthropogenic contaminants on genetic diversity [53, 54, 55], but also many cases where metal contamination had no or only small effect on genetic diversity, e.g. in the earthworm *Dendrobaena octaedra* [43, 56, 57], enchytraeid worm *Cognettia sphagnetorum* [58], water flea *Daphnia longispina* [59], springtail *Orchesella cincta* [60] and woodlice *Porcellionides sexfasciatus* [61]. For the beetle, *Staphylinus erythropterus,* higher genetic diversity was found in populations from metal contaminated sites than from reference sites [62]. In light of this, it is possible that when species diversity is diminished in metal contaminated environments, the genetic diversity of the remaining species could become more important for ecosystem functioning, in a similar way as species diversity is more important in species-poor, rather than in species-rich communities [24].

Decomposition is critical for ecosystem functioning [63] and soil invertebrates are important decomposers [46], but to our knowledge, whether the genetic diversity of soil invertebrates affects decomposition processes has not been studied before. The epigeic earthworm *Dendrobaena octaedra* is a key decomposer in boreal coniferous forests [47, 64]. *D. octaedra* reproduces through apomictic parthenogenesis [65, 66, 67] which means that the offspring are clones of their parent (Mustonen et al. submitted). Nevertheless, it has high genetic diversity, both in uncontaminated and metal contaminated habitats [43, 55, 56].

These features make *D. octaedra* a good species to study the effect of genetic diversity on decomposition processes, allowing additional understanding of the diversity-functioning relationship in terrestrial ecosystems.

We established a year-long microcosm experiment simulating two growing seasons in which the microcosms contained *D. octaedra* with either low diversity (clones) or high diversity (different genotypes). The experiment was done in both uncontaminated and moderately Cu-contaminated soil. Moreover, half of the microcosms contained a simple community of other soil mesofauna (no intentionally added other decomposers) and the other half of the microcosms contained a diverse community (added another earthworm species, enchytraeds and microarthropods). CO_2 production was used as a proxy for soil biological activity and NH_4-N concentration as proxy for decomposition efficiency. Cocoon production, growth and mortality of the earthworms were used as measures of how well the earthworms thrived in microcosms. Gene expression of five genes were used as a proxy for metabolic activity and expression of one gene involved in metal tolerance was used as a measure of the effect of Cu-contamination on the earthworms. Using our microcosm experiment we asked 1. how does genetic diversity of one key-species, *Dendrobaena octaedra*, affect decomposition processes, and 2. is the response different in uncontaminated soil than in Cu-contaminated soil, and 3. does the diversity (low or high) of other decomposers change the response? We hypothesized that decomposition activity and nutrient mineralization are higher in microcosms with high genetic diversity, and the difference between diversity treatments is larger in a stressful environment (i.e. metal contaminated soil). We also hypothesized that higher species diversity of other decomposers could mask the differences between low diversity and high genetic diversity of the key-species, since there are more functional groups contributing to the decomposition process.

MATERIALS AND METHODS

Sampling the earthworms and establishing the cultures

Dendrobaena octaedra were collected from Harjavalta (South-West Finland, 61°18'50''N, 22°08'30''E) ca. 1 km from a smelter which is the source of Cu (~823 mg/kg) and Zn (~474mg/kg) contamination in the soil [68]. Earthworms were collected by hand in September 2012 and transferred to the laboratory in buckets with soil from the sampling site. Cultures were established in the laboratory, each from a single juvenile or sub-adult earthworm, in glass jars (Ø 8 cm, with perforated lids) containing uncontaminated organic-rich soil and horse manure (for nutrition). All cultures were maintained at 15 °C and the soil was changed every few months for two years. When needed, the cultures were transferred to larger plastic jars (Ø 13 cm, with perforated lids) to avoid overcrowding. Because *D. octaedra* reproduces through apomictic parthenogenesis, all offspring of the founder earthworm, and their offspring, are assumed to be genetic clones. One earthworm from each culture was used for genotyping to ensure that dif-

ferent genotypes were used in the experiment (Mustonen et al, unpubl.; see Additional file 1, for the genotypes used as well as the procedure for DNA extraction and genotyping).

The experimental setup

Microcosms were established and the experiment was started in November 2014. We examined the effect of *D. octaedra* genetic diversity on decomposition activity using two different treatments: low diversity (LD; added *D. octaedra* were clones) and high diversity (HD; added *D. octaedra* were different genotypes). In the low diversity treatments, four earthworms of the same genotype were put into each microcosm. Three different cultures (clone families) were used, and genotyping revealed that two of the clone families had the same multilocus genotype based on five microsatellite markers (See Additional file 1). Four or six replicates of the low diversity treatments were made (six replicates when using the clone family that differed from the other two). In the high diversity treatments, four earthworms of different genotypes were put into each microcosm. Three different genotype-compositions were used and there were four replicates of each (See Additional file 1).

Half of the microcosms contained uncontaminated soil and half contained moderately Cu-contaminated soil (100 mg kg^{-1} dry mass). Soil used in the experiment was collected from an uncontaminated mature spruce forest in Jyväskylä and some fallen birch leaves were shredded and mixed well with the soil for food (pH = 5.2; organic matter content = 79,5%). For half of the soil-leaf mixture, we mixed 845.4 mg of copper chloride ($CuCl_2$ H_2O) in 6000 ml of water and mixed it thoroughly with 6 kg soil (wet mass) to achieve a contamination level of Cu 100 mg kg^{-1} (dry soil). After metal addition, pH of the soil decreased to 5.1. The spiked soil was incubated for 7 days at 15°C before the experiment. Pure water was added to the soil for the uncontaminated treatment. During the experiment, water was added to the microcosms about every other week (except during "winter") to replace what was lost from evaporation.

Additionally, we included either low or high diversity of other decomposer animals. Low diversity microcosms derived their few taxa with living earthworms introduced to the microcosms. For the high diversity condition, one *D. octaedra* individual was replaced with one *Dendrodrilus rubidus* earthworm. Also, ten individuals of the enchytraeid *Cognettia sphagnetorum* were added (previously extracted from organic rich soil using a standard wet funnel method). In addition, samples of soil microarthropods (extracted from soil samples equivalent to the soil used in the microcosms) were added to microcosms with the diverse community. Together with the enchytraeids and microarthropods, associated microfauna (nematodes, tardigrades and rotators) were also included. Altogether there were 104 microcosms. The experimental setup is represented in Table 1.

Microcosms were kept in a climate chamber with changing temperature to mimic different seasons. The experiment was started with autumn, with temperature first set to 12 ^{o}C and gradually decreasing to 1 ^{o}C over four weeks to

winter. Winter lasted three weeks with a steady temperature of 0.5 °C. Temperature increased in spring to 12 °C over three weeks. Summer lasted 11 weeks, during which temperature increased from 13 to 14 °C, one week each, and then was held at 15 °C for seven weeks before decreasing back to 14 and 13 °C, one week each. After the first summer, half of the microcosms were destructively sampled and a second growing season was continued with the earthworms from the remaining microcosms (following the same temperature regime), but with new soil and new individuals of other decomposer animals. After the first growing season, we discovered that the earthworms in the microcosms located on the lowest level of the climate chamber had died, so these microcosms (containing Cu-contaminated soil, *D. octaedra* and high diversity of other decomposer animals) were repeated.

Collecting data

NH_4-N concentration was measured from the microcosms that were destructively sampled after the first growing season and at the end, as well as from the original soil used in the experiment: soil was extracted with 2 M KCl-solution, and extracted samples were analyzed by Nablabs Oy (Jyväskylä) using SFS-EN ISO 11732:2005 method. CO_2 production was measured throughout the experiment with a Calanus UniQuant carbon analyzer, eight measurements during growing season; twice in the autumn and spring, and about every two weeks in the summer. Production of CO_2 was measured by first determining the base level of CO_2 by injecting 1 ml of air from the microcosms to the carbon analyzer, then closing the microcosm and keeping it air-tight for 1.5 h (during which the microcosms were back in the climate chamber). Afterwards, another 1 ml sample of air was taken and analyzed for CO_2. CO_2 production was calculated from the difference between the two samples taking into account the volume of each microcosm and the amount of soil (µg/g/h). Data from all measurements (throughout the growing season) were analyzed together.

Biomass of the earthworms was measured at the beginning, after the first growing season and at the end of the experiment, and in the analyses initial biomass was used as a co-variant. Biomass was used to measure the growth of the earthworms, thus the years are analysed separately. Cocoon production (all cocoons, including cocoons of *D. rubidus* in the high species diversity microcosms), numbers of other decomposer animals, as well as mortality of *D. octaedra* were recorded after the first growing season and at the end of the experiment. Mortality was high for *D. rubidus*, with only a few individuals found alive after the first growing season and at the end of the experiment.

Gene expression of five genes involved in metabolism and one gene involved in metal tolerance was measured from the *D. octaedra* after the first growing season and at the end of the experiment. Earthworms were removed from the microcosms and after weighing, put in glass jars with moist paper towel for two days to allow them to empty their guts. Paper towel was changed after one day. A piece of tissue (ca. 5 mm) from the anterior end of each earthworm was cut with a scalpel, placed in separate 1.5 ml microcentrifuge tubes

and immediately frozen in liquid nitrogen. Samples were stored at -80 °C until RNA extraction. Gene expression was measured from aldo/keto reductase (AkRed), chymotrypsin inhibitor (ChymInh), dual specifity phosphatase 14 (DualPhos), glucose-regulated protein 94 (GlucReg), pancreatic amylase (PanAmy) and metallothionein (MT). Also, 18S and 28S ribosomal gene expression was measured to be used as potential reference genes. All primers, except those for MT, 18S and 28S [described in 68], were designed based on a draft transcriptome of *D. octaedra,* in which GO-terms were assigned to sequences (shared by M. Holmstrup, unpublished, Additional file 2).

RNA extraction and qPCR protocol

For RNA extraction, we used the Aurum Total RNA mini kit (Bio-Rad) following the manufacturer's protocol including DNase I treatment. The concentration of the extracted RNA was measured using the Qubit RNA Assay Kit and Qubit Fluorometer (Invitrogen, Turner BioSystems). For cDNA synthesis, we used the iScript cDNA synthesis kit (Bio-Rad) following the manufacturer's protocol, using 35 ng of RNA per reaction. After synthesis, cDNA was diluted 1:5. Real-time quantitative polymerase chain reactions (qPCR) were performed using IQ SYBR green supermix (Bio-Rad). qPCR conditions had been optimized and efficiencies had been checked with a dilution series (5 points, 10-fold dilutions) (Additional file 2), prior to this study. In each reaction we used 1 µl of cDNA template and 0.5 µM of each primer in a final reaction volume of 20 µl (for the efficiency estimates) or 10 µl (for the gene expression measurement). Three replicate reactions for each sample were prepared and an inter-run calibrator was used. For all reactions, we used a CFX96 C1000 Touch Thermal Cycler (Bio-Rad) with the following protocol: 94°C for 2 minutes and then 40 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 15 sec followed by a plate read. A melt curve analysis was done at the end of each amplification reaction to ensure a single amplification product.

Data analysis

Raw gene expression data and stability of the reference genes was checked with qBase+ (Biogazelle). 18S and 28S turned out to be unsuitable as reference genes for this dataset, and so NORMA-Gene [69] was used for normalization. NH_4-N concentration, CO_2-production, cocoon production, earthworm biomass, mortality and the normalized gene expression data were analyzed in IBM Statistics SPSS 20. Conformity to a normal distribution was tested using a Kolmogorov-Smirnov test or Shapiro-Wilk test when appropriate, and homogeneity of variances was examined with Levene's test. Data was log-transformed when necessary to meet those assumptions, but it did not help in all cases. Our main focus was to test the differences between low (LD) and high genetic diversity (HD) treatments, which was tested in general (over all conditions combined) and also separately for the different conditions (uncontaminated or Cu-contaminated soil, microcosms with low or high diversity of other soil animals). We also tested the data overall (combining the data from the whole experiment) and for the

first and the second year separately. Differences between the LD families and between the HD groups (different genotype compositions) were also tested, and since there were only two genotypes in LD (family 1 had one genotype and families 2 and 3 had another), the difference between LD and HD was also tested for the two genotypes separately. The overall differences between the uncontaminated and contaminated soil and microcosms with low or high diversity of other soil animals were also tested. One-way ANOVA or Kruskal-Wallis tests were used.

RESULTS

Soil mineral nitrogen concentration

In general (when the conditions were combined) there was no difference between LD and HD treatments in soil NH_4-N concentration, both overall and when the first year and second year were analyzed separately (Table 2). When analyzing the different conditions separately, the NH_4-N concentration was higher in HD than in LD in the first year in contaminated soil with high diversity of other decomposer animals (Table 2, Figure 1).

When comparing the clone families, soil NH_4-N concentration was higher in the presence of clone family 1 (LD1) than in the presence of clone families 2 or 3 (LD2 and LD3; Additional files 3 and 4). We also compared the HD treatment to the two genotypes in LD separately: when comparing HD to LD1, NH_4-N concentration was higher in LD than HD, but when HD was compared to LD2 and LD3, the result was the opposite (Additional files 3 and 4). The overall response of the different LD families and the HD treatment to the different conditions (uncontaminated or contaminated soil, low and high diversity of other decomposers) is represented in Additional file 5: the responses of families LD1 and LD2 were similar (decrease in NH_4-N in contaminated soil compared to uncontaminated) in microcosms with high diversity of other decomposer animals, but a different response (increase in NH_4-N in contaminated soil compared to uncontaminated) was shown by LD3 (even though LD2 and LD3 have the same genotype). Response of the HD treatment differed from the LD families in microcosms with low diversity of other decomposer animals, but was similar to response of LD3 in microcosms with high diversity of other decomposer animals. There was no difference in NH_4-N between uncontaminated and contaminated soil or between low and high diversity of other decomposer animals (Additional file 3).

CO_2 production

In general, more CO_2 was produced in LD than in HD treatments (Table 3; mean in LD: 3.84 μg/g/h, mean in HD: 3.56 μg/g/h). However, when analyzing the data in the different years of the experiment, the difference was only significant in the first year, and more specifically, only in the treatment with

uncontaminated soil and high diversity of other decomposer animals (Table 3, Figure 2). More CO_2 was produced in the first year than in the second year (X^2= 17.344, df=1, p<0.001, Figure 2).

There was no difference between the three LD families or the three HD groups in CO_2 production (Additional file 3). HD was also compared to the two genotypes in LD separately, and we found that more CO_2 was produced in LD2 and LD3 (mean: 3.79 μg/g/h) than in HD (mean: 3.56 μg/g/h), but there was no difference when HD was compared to LD 1 (Additional file 3). When comparing the different conditions, more CO_2 was produced in contaminated soil than in uncontaminated soil, but there was no difference between conditions with low and high diversity of decomposer animals (Additional file 3). The overall CO_2 production response of the different LD families and the HD treatment to the different conditions (uncontaminated or contaminated soil, with low and high diversity of other decomposer animals) is shown in Additional file 6: the responses of families LD1 and LD2 were similar (only a slight increase in CO_2 production in contaminated soil compared to uncontaminated) in microcosms with high diversity of other decomposer animals, but LD3 showed a different response (large increase in CO_2 production in contaminated soil compared to uncontaminated). Response of the HD treatment differed from the LD families in microcosms with low diversity of other decomposer animals, but was similar to response of LD1 and LD2 in microcosms with high diversity of other decomposer animals.

Earthworm growth and cocoon production

In general, there was no difference in the biomass of the earthworms between LD and HD treatments in the first year, but in the second year, the earthworm biomass was higher in the HD treatments (Table 4, Additional file 7). In the first year, no difference was found between LD and HD treatments when the different conditions were analyzed separately, but in the second year, HD earthworms had higher biomass in the contaminated soil with high diversity of other decomposer animals (Table 4, Additional file 7).

There was no difference in biomass between the LD families in the first year, but in the second year, earthworms from LD1 had higher biomass than those in LD2 (LSD: mean difference: 0.045, p<0.001) and those in LD3 (LSD: mean difference 0.062, p<0.001)(Additional files 8 and 9). When comparing HD to LD1, there were no differences in earthworm biomass in either year, but when comparing HD to LD2 and LD3, there was higher earthworm biomass in HD in the second year (Additional files 8 and 9). When comparing the different conditions, there were also differences in earthworm biomass between uncontaminated soil and contaminated soil and between microcosms with low or high diversity of other decomposer animals (in the second year). In these cases, earthworms had higher biomasses in contaminated soil with high diversity of other decomposer animals (Additional file 8, Figure 2).

There were no differences in cocoon production between LD or HD treatments overall or when the first year and second year were analyzed separately,

both in general and when the different conditions were analyzed separately (Additional file 10). More cocoons were produced in presence of LD1 than in LD2 or 3 (Additional files 3 and 11). When comparing HD to LD1, there was higher cocoon production in LD, but no difference in cocoon production when comparing HD to LD2 and LD3 (Additional file 3). There was no difference between uncontaminated and contaminated soil conditions, or between low or high diversity of other decomposer animals (Additional file 3).

Mortality and other species

Mortality of *D. octaedra* did not differ between LD and HD treatments overall, but it did in the first year when the years were analyzed separately. Specifically, there was higher mortality in HD treatments with uncontaminated soil and low decomposer animal diversity (Additional file 12). Mortality did not differ between different LD families, between HD and the different LD genotypes, between uncontaminated and contaminated soil conditions, or between and low and high decomposer animal diversity (Additional file 3).

Numbers of the other decomposer animals (enchytraeids, collembola, and mesostigmatid and oribatid mites) in the microcosms after the first and second years are shown in Additional file 13. Animal numbers clearly differed between the low and high diversity conditions.

Gene expression

In general, gene expression differed between LD and HD treatments in only one of the genes, ChymInh (Table 5), the expression being higher in HD (Figure 3). The difference in ChymInh expression was only significant in the first year and not the second, when years were analyzed separately (Additional file 14). In the second year, expression of the gene AkRed was higher in the LD treatment (Table 5, Additional file 14).

Examining the different conditions, HD treatments showed higher expression of ChymInh in uncontaminated soil with low diversity of other decomposer animals, both overall and when the years were analyzed separately. In uncontaminated soil with high diversity of other decomposer animals, LD treatments showed higher expression of AkRed (overall), DualPhos (overall and in the second year) and MT (overall and in the first year) (Table 5, Additional files 15, 16 and 17). In contaminated soil with low diversity of other decomposer animals there were no differences between LD and HD treatments overall, but expression of AkRed was higher in the LD treatment in the second year, and expression of ChymInh and DualPhos were higher in the HD treatments in the first year and the second year, respectively (Table 5, Additional files 15, 16 and 17).

Comparing the different clone families, expression of AkRed was higher in LD1 than in LD2 and LD3 (Post hoc LSD $p < 0.001$ for both) and expression of PanAmy was lower in LD1 than in LD2 (Post hoc LSD $p=0.001$). When comparing HD to LD2 and LD3, there were no differences in gene expression, but when comparing HD to LD1 there was higher expression of AkRed in LD, and

higher expression of PanAmy and ChymInh in HD (Additional files 3 and 18). The overall gene expression response (all genes combined) of the different LD families and HD treatment to the different conditions (uncontaminated or contaminated soil, with low or high diversity of other decomposer animals) is represented in Additional file 19: the responses of families LD1 and LD2 were similar (only a slight increase in gene expression in contaminated soil compared to uncontaminated), but LD3 showed a different response (large increase in gene expression in contaminated soil compared to uncontaminated) when there was high diversity of the other decomposer species.

When comparing the different conditions, expression of DualPhos was higher in uncontaminated than in contaminated soil and expression of MT was higher in contaminated than in uncontaminated soil (Additional files 3 and 15). Expression of both ChymInh and MT was higher in high diversity of the other decomposer animals than in low (Additional files 3 and 15).

DISCUSSION

Genetic diversity has a positive effect on ecosystem functioning, as does species diversity, both in plants [10, 11, 12, 13, 14, 16, 15, 17] and in some animals [18, 19]. To our knowledge, however, the effect of genetic diversity on decomposition processes, which are an important part of terrestrial ecosystem functioning, has not been studied before. Using a microcosm experiment, we investigated whether higher genetic diversity in a key decomposer species, *Dendrobaena octaedra*, leads to higher decomposition activity than does lower genetic diversity, and if higher genetic diversity of *D. octaedra* is more important in stressful environmental conditions (metal contaminated soil). We also wanted to see what effect the diversity of other decomposer animals might have on the relationship between genetic diversity of *D. octaedra* and decomposition processes.

We found some indications that higher genetic diversity of *D. octaedra* has a positive effect on decomposition processes and that high diversity of the earthworms is especially beneficial in stressful conditions: in contaminated soil, NH_4-N concentration was higher in the high diversity treatment (only in the first year). Moreover, earthworms in the high diversity treatments seemed to cope well (with higher biomass) in contaminated soil, and had poorer condition in uncontaminated soil (evidenced by lower soil CO_2 production and higher mortality). Most of the differences between high and low genetic diversity treatments appeared, however, only when there was a diverse community of other decomposer animals present in the soil, indicating that high species diversity influences how genetic diversity of a key decomposer affects soil processes (see below). Interestingly, it seems that the specific genotype or lineage of the earthworms matters more than does genetic diversity *per se*. For many of the parameters we studied, the effect of the diversity treatment was dependent on whether the high diversity group was compared to one or the other of the genotypes in the low diversity treatments.

We hypothesized that the presence of diverse community of other decomposer animals might prevent us from detecting differences between low genetic diversity and high genetic diversity treatments on decomposition processes, since there are more functional groups present when there are more species in the community, but that did not seem to be the case. Quite often there was a difference between LD and HD treatments only in the presence of diverse decomposer community (e.g. higher NH_4-N concentration in HD in contaminated soil, or higher CO_2 production in LD in uncontaminated soil). In contrast to our expectation, higher species diversity seemed to make the differences between low and high genetic diversity treatments more apparent. Since the microcosms that had a diverse decomposer community were more similar to the natural conditions (i.e. having myriad interactions, both direct and indirect, between different decomposer groups) than the microcosms in which only few decomposer animals were present, these results might be more generally applicable for understanding the role of genetic diversity on decomposition processes in natural habitats. However, inferences about field conditions based on a controlled microcosm-experiment such as this should be made with caution.

Species diversity has often been shown to be beneficial for ecosystem functioning [1, 2, 3, 4, 5], but in our study the only clear positive effect of adding more species to the microcosms on any parameter measured was the increased growth of the earthworms (measured from the biomass) in those microcosms. This was surprising because the additional species were expected to increase nitrogen mineralization and biological activity, since previous studies have shown that more functional groups can lead to more efficient decomposition processes [23, 24, 25, 26]. Our microcosm results support conclusions made by some other researchers, namely that the presence of key decomposers is more important for ecosystem functioning in soil than is the diversity of the decomposers [25, 26]. On the other hand, since our microcosms had limited structural diversity (homogeneous soil) and quite few decomposer animal species, it might have been difficult to detect any impact of the additional decomposer species. A factor that could have influenced our findings was the high mortality of the other introduced earthworm species, *D. rubidus,* in the microcosms of high decomposer animal diversity. *D. rubidus* is another epigeic earthworm, possibly with slightly different feeding strategy than *D. octaedra* [70], allowing a possibility for increased impact on decomposition processes through complementation. The high mortality of *D. rubidus* might explain the higher biomass of *D. octaedra* in the microcosms with high diversity of decomposer animals: when the *D. rubidus* individuals died, there were more resources available for the *D. octaedra*.

We found some positive effects of higher genetic diversity on decomposition in contaminated conditions. It seems that the earthworms also thrived well in contaminated soil, with higher growth and soil CO_2-production in contaminated than in uncontaminated soil. Li and colleagues [40] found that in algal communities, species diversity was important for productivity in cadmium-polluted conditions, and Cd-tolerant species were doing especially well. Our

results showed that the positive effect of diversity can apply also at the genetic level and in terrestrial ecosystems. The earthworms in our study originated from a site where metals have accumulated in the soil for many decades due to emissions from a Cu-Ni smelter [71]. Adaptation of *D. octaedra* to these conditions is likely [68]. Moderate levels of Cu-contamination, such as that used in our experiment, can have positive effects on earthworms, e.g. on growth and reproduction of *Aporrectodea tuberculata* [72] and on our study species, *D. octaedra* [73, 74].

For the gene expression measurements, we selected genes expected to be involved in metabolism, and we also included a gene for the metal-tolerance protein metallothionein (MT, described in Mustonen et al. 2014). MT has been linked to adaptation of *D. octaedra* to metal contaminated soil [68, 75]. Overall expression of MT was higher in metal contaminated conditions, which was expected. But, when considering the genetic diversity treatments, expression of MT was higher in low diversity than high diversity treatments in uncontaminated soil with high diversity of other decomposer animals. The difference between low and high diversity was probably not related to the function of MT as a response to Cu exposure, since the difference was found only in the uncontaminated soil. In our previous study [68], we found that there is high variation among individuals of *D. octaedra* in expression of MT, in contaminated as well as in uncontaminated soil. It could be that the genotypes in our low diversity treatment happen to be genotypes that express MT at a higher level in uncontaminated soil, whereas the genotypes in high diversity express MT at a lower level, or have varying expression responses, leading to the overall higher expression in the low diversity treatment.

The specific function of MT and the functions of the other gene products have not been experimentally confirmed in *D. octaedra*. Therefore, we relied on the original GO-terms assigned to the transcriptome-sequences, and we can only speculate about functional responses when examining the results of our gene expression comparisons. The protein chymotrypsin inhibitor inhibits the production of chymotrypsin, a digestive enzyme involved in protein degradation [76], but also other functions have been described for this protein, e.g. involvement in the immune response [77]. Expression of the chymotrypsin inhibitor gene (ChymInh) was most consistent in showing differences between the low diversity and high diversity treatments, with higher gene expression in worms from the high diversity microcosms. Whether this response has a specific effect on decomposition, however, is not clear.

Of the other metabolic genes studied, we observed only a few differences between the low diversity and high diversity treatments, and sometimes only under certain conditions, preventing any generalization of the gene expression patterns. For example, expression of aldo/ketoreductase (AkRed) was higher in the low diversity treatment, more specifically in low diversity family 1 (LD1), than in the high diversity treatments. AkRed is an enzyme which is expected to be involved in different oxidation-reduction processes in metabolism, including digestion [78], but which can also participate in other processes, e.g. detoxifica-

tion of pharmaceuticals [79]. Phosphatases are involved in many metabolic processes, including digestion [80], but the phosphatase gene we measured, dual-specificity phosphatase 14 (DualPhos) has been connected to immune reaction in mice [81], with enhanced immune response in mice deficient in dual-specificity phosphatase 14. Expression of this gene was lower in low diversity treatments in uncontaminated soil with high diversity of other decomposer animals, but higher in high diversity treatments in contaminated soil with low diversity of other decomposer animals. Pancreatic amylase (PanAmy) is involved in digestion [80], and expression of this gene was lower in low diversity family 1 (LD1) than in high diversity (HD). Additional studies into the function of these genes in *D. octaedra* are required before we can make conclusions on what these differences mean at the functional level. It also has to be remembered that we might have observed different gene expression responses if we had studied other tissues from the earthworms.

Although general conclusions based on the gene expression results are hard to make, the results show some trends similar to what we found using the other functional response parameters, soil NH_4-N concentration and CO_2-production. The genotype used in the low diversity treatment has a large impact on the results: no differences were found in gene expression between high diversity and low diversity treatments when including only low diversity families 2 and 3 (LD2 and LD3, which had the same genotype), but differences were evident when comparing the high diversity treatments to low diversity family 1 (LD1, a different genotype from LD2 and LD3). Interestingly, how the families responded to the different conditions also differed. Although family 1 and family 2 were different genotypes, their response to the different conditions was very similar in NH_4-N concentration, CO_2 production and gene expression; whereas the response of family 3 was different, especially in the presence of a diverse animal community (see Additional files 5, 6 and 19). This suggests that families 2 and 3, which were reared from different founding individuals are not, in fact, genetically identical (even though they are identical at the five microsatellite loci studied here). Previously, we have found considerable differences in gene expression even among individuals sharing the same genotype from the same founder (Mustonen et al., submitted). Mechanisms allowing different gene expression responses in clones would be an interesting subject for further studies.

Although we strived to control the experimental conditions as well as possible, there were some unanticipated problems. Microcosms with contaminated soil and high diversity of other decomposer animals had to be repeated due to a technical problem with the climate chamber. All replicates of both low diversity and high diversity treatments in this condition were repeated, and the same temperature regime and handling of the microcosms was used, so differences between the original and repeat are likely to be minimal. However, differences in the response between the conditions (uncontaminated and contaminated soil; with low and high diversity of other decomposer animals) might be influenced by slight differences between the repeat and original microcosms. Nevertheless,

our comparison of the low diversity and high genetic diversity treatments were not affected.

Our experiment lasted two growing seasons ("years"), and data was analyzed overall and with the data from the first year and second year separated. In some cases there was a significant difference between low diversity and high diversity treatments after the first year but not after the second year (NH_4-N concentration, CO_2 production, earthworm mortality, expression of some genes). The conditions in the microcosms were the same for the first year and the second year; soil and the introduced decomposer animals were changed, but the *D. octaedra* were the same individuals in both years. Thus, the second year was a repeat of the first year in every way except for the age of *D. octaedra*: in the first year they were juveniles or subadults and in the second year they were fully grown adults. In many cases we found differences between the diversity treatments in the first year, but not in the second. Since we expected the responses would be the same in both years, we can question whether the significant results in the first year are only due to chance. However, senescence of the older earthworms in the second year might have affected significantly their impact on soil processes, and reduced any differences that were evident between low and high genetic diversity treatments in the first year. There was lower CO_2 production during the second year than the first year, which could have been at least partly affected by lower activity of the older earthworms.

CONCLUSIONS

Higher genetic diversity of key decomposer animal species could be beneficial for the functioning of terrestrial ecosystems through more efficient decomposition processes, especially in contaminated conditions. But particular genotypes, well-adapted to the stressful conditions can also maintain ecosystem function even when there is low genetic diversity. Anthropogenic contaminants can reduce species diversity [34, 35, 36, 37], and in these environments ecosystem function depends on the remaining species and genotypes. Higher standing genetic diversity makes it more likely that some genotypes are high-functioning in the changed environmental conditions, and provide a means for adaptation via natural selection. Our microcosm study provides some insight into the role of genetic diversity in decomposition processes (i.e. ecosystem functioning), but strong conclusions are not possible since the differences between the low and high genetic diversity were few and often present in only one of the growing seasons studied. The relationship between genetic diversity and ecosystem functioning should be studied with additional populations, including those that have not previously experienced metal contaminated soils.

Acknowledgements

The authors are grateful to Mustapha Boucelham, M.Sc. for help in processing animal and soil samples and to Martin Holmstrup for sharing a draft transcriptome of *D. octaedra.*

REFERENCES

1. Gravel,D., Bell, T., Barbera, C., Bouvier, T., Pommier, T., Venail, P., Mouquet, N. Experimental niche evolution alters the strength of the diversity–productivity relationship. Nature 2011; 469: 89-92.
2. Hooper, D.U., Adair, E.C., Cardinale, B.J., Byrnes, J.E.K., Hungate, B.A., Matulich, K.L., Gonzales, A., Duffy, J.E., Gamfeldt, L., O'Connor, M.I. A global synthesis reveals biodiversity loss as a major driver of ecosystem change. Nature 2012; 486:105-108.
3. Harrison, P.A., Berry, P.M., Simpson, G., Haslett, J.R., Blicharska, M., Bucur, M., Dunford, R., Egoh, B., Garcia-Llorente, M., Geamănă, N., Geertsema, W., Lommelen, E., Meiresonne, L., Turkelboom, F. Linkages between biodiversity attributes and ecosystem services: A systematic review. Ecosystem services 2014; 9: 191-203.
4. Duncan, C., Thompson, J. R., Pettorelli, N. The quest for a mechanistic understanding of biodiversity – ecosystem services relationships. Proc R Soc B 2015; 282: 20151348.
5. Evans, E. W. Biodiversity, ecosystem functioning, and classical biological control. Appl Entomol Zool 2016; 51:173-184.
6. Bassar, R. D., Marshall, M. C., Lopez-Sepulcre, A., Zandona, E., Auer, S. K., Travis, J., Pringle, C. M., Flecker, A. S., Thomas, S. A., Fraser, D. F., Reznick, D. N. Local adaptation in Trinidadian guppies alters ecosystem processes. PNAS 2010; 107: 3616-3621.
7. Matthews, B., Narvani, A., Hauch, S., Nonaka, E., Peter, H., Yamamichi, M., Sullam, K. E., Bird, K. C., Thomas, M. K., Hanley, T. C., Turner, C. B. Toward an integration of evolutionary biology and ecosystem science. Ecol Letters 2011; 14: 690-701.
8. Barraclough, T. G. How do species interactions affect evolutionary dynamics across whole communities? Annu Rev Ecol Syst 2015; 46:25-48.
9. Harmon, L. J., Matthews, B., Des Roches, S., Chase, J. M., Shurin, J. B., Schluter, D. Evolutionary diversification in stickleback affects ecosystem functioning. Nature 2009; 458: 1167-1170.
10. Hughes, A. R., Inouye, B. D., Johnson, M. T. J., Underwood, N., Vellend, M. Ecological consequences of genetic diversity. Ecol Letters 2008; 11: 609-623.
11. Reusch, T. R., Hughes, A. R. The Emerging Role of Genetic Diversity for Ecosystem Functioning: Estuarine Macrophytes as Models. Estuar Coast 2006; 29: 159-164.

12. Avolion, M. L., Chan, C. C., Weis, J. J., Smith, M. D. The effect of genotype richness and genomic dissimilarity of *Andropogon gerardii* on invasion resistance and productivity. Plant Ecol Divers 2014; 8:61-71.
13. Hughes, A. R. Genotypic diversity and trait variance interact to affect marsh plant performance. J of Ecol 2014 102: 651-658.
14. Schöb, C., Kerle, S., Karley, A. J., Morcillo, L., Pakeman, R. J., Newton, A. C., Brooker, R. W. Intraspecific genetic diversity and composition modify species-level diversity-productivity relationships. New Phytol 2014; 205: 720-730.
15. Prieto, I., Violle, C., Barre, P., Durand, J., Ghesquiere, M., Litrico, I. Complementary effects of species and genetic diversity on productivity and stability of sown grasslands. Nature Plants 2015; 1: Article number: 15033.
16. Whitlock, R. Relationships between adaptive and neutral genetic diversity and ecological structure and functioning: a meta-analysis. J of Ecol 2104;102: 857-872.
17. Wymore, A. S., Keeley, A. T. H., Yturralde, K. M., Schroer, M. L., Propper, C. R., Whitman, T. G. Genes to ecosystems: exploring the frontiers of ecology with one of the smallest biological units. New Phytol 2011;191: 19-36.
18. Aguirre, J. D., Marshall D. J. Genetic diversity increases population productivity in a sessile marine invertebrate. Ecology 2012; 93: 1134-1142.
19. Johnson, D. W., Freiwald, J., Bernardi, G. Genetic diversity affects the strength of population regulation in a marine fish. Ecology 2016; 97:627-639.
20. Hättenschwiler, S., Tiurnov, A. V., Scheu, S. Biodiversity and litter decomposition in terrestrial ecosystems. Annu Rev Ecol Syst 2005; 36:191-218.
21. Gessner, M.O., Swan, C. M., Dang, C. K., McKie, B. G., Bardgett, R. D., Wall, D. H., Hättenschwiler, S. Diversity meets decomposition. Trends Ecol Evol 2010; 25: 372-380.
22. Bardgett, R. D., van der Putten, W. H. Belowground biodiversity and ecosystem functioning. Nature 2014; 515: 505-511.
23. Laakso, J., Setälä, H. Sensitivity of primary production to changes in the architecture of belowground food webs. Oikos 1999; 87:57-64.
24. Ekschmitt, K., Klein, A., Pieper, B., Wolters, V. Biodiversity and functioning of ecological communities - why is diversity important in some cases and unimportant in others? J Plant Nutr Soil Sci 2001; 164:239-246.
25. Cragg, R. G., Bardgett, R. D. How changes in soil faunal diversity and composition within a trophic group influence decomposition processes. Soil Biol & Biochem 2001; 33: 2073-2081.
26. Heemsbergen, D. A., Berg, M. P., Loreau, M., van Hal, J. R., Faber, J. H., Verhoef , H. A. Biodiversity effects on soil processes explained by interspecific functional dissimilarity. Science 2004; 306: 1019-1020.
27. Eisenhauer, N., Schädler, M. Inconsistent impacts of decomposer diversity on the stability of aboveground and belowground ecosystem functions. Oecologia 2011; 165:403-415.

28. Scheu, S., Schlitt, N., Tiunov, A. V., Newington, J. E., Jones, T. H. Effects of the presence and community composition of earthworms on microbial community functioning. Oecologia 2002; 133:254-260.
29. Zimmer, M., Kautz, G., Topp, W. Do woodlice and earthworms interact synergistically in leaf litter decomposition? Funct Ecol 2005;19: 7-16.
30. Adejuyigbe, C.O., Tian, G., Adeoye, G. O. Microcosmic study of soil microarthropod and earthworm interaction in litter decomposition and nutrient turnover. Nutr Cycl in Agroecosyst 2006; 75: 47-55.
31. Eisenhauer, N., Reich, P. B., Isbell, F. Decomposer diversity and identity influence plant diversity effects on ecosystem functioning. Ecology 2012; 93: 2227-2240.
32. Wagg, C., Bender, S. F., Widmer, F., van der Heijden, M. G. A. Soil biodiversity and soil community composition determine ecosystem multifunctionality. Proc. Natl. Acad. Sci. USA 2014;111: 5266-5270.
33. Jung, J., Philoppot, L., Park, W. Metagenomic and functional analyses of the consequences of reduction of bacterial diversity on soil functions and bioremediation in diesel-contaminated microcosms. Scientific Reports 2016; 6: Article number 23012.
34. Islam, Md. S., Tanaka, M. Impacts of pollution on coastal and marine ecosystems including coastal and marine fisheries and approach for management: a review and synthesis. Mar Pollut Bull 2004; 48: 624-649.
35. Johnston, E. L., Roberts, D. A. Contaminants reduce the richness and evenness of marine communities: A review and meta-analysis. Environ Pollut 2009; 157:1745-1752.
36. Sutton, N. B., Maphosa, F., Morillo, J. A., Al-Soud, W. A., Langenhoff, A. A. M., Grotenhuis, T., Rijnaarts, H. H. M., Smidt, H. Impact of Long-Term Diesel Contamination on Soil Microbial Community Structure. Appl Environ Microbiol 2013; 79: 619-630.
37. Quero, G. M., Cassin, D., Botter, M., Perini, L., Luna, G. M. Patterns of benthic bacterial diversity in coastal areas contaminated by heavy metals, polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs). Front Microbiol 2015; 6: 1053
38. Johnston, E. L., Mayer-Pinto, M., Crowe, T. P. Chemical contaminant effects on marine ecosystem functioning. J of Appl Ecol 2015; 52:140-149.
39. Fariña, J. M., Castilla, J. C., Ojeda, F. P. The "idiosyncratic" effect of a "sentinel" species on contaminated rocky intertidal communities. Ecol Appl 2003; 13:1533-1552.
40. Li, J., Duan, H., Li, S., Kuang, J., Zeng, Y., Shu, W. Cadmium pollution triggers a positive biodiversity–productivity relationship: evidence from a laboratory microcosm experiment. J of Appl Ecol 2010; 47: 890-898.
41. Bengtsson, G., Nordstrom, S., Rundgren, S. Population-density and tissue metal concentration of lumbricids in forest soils near a brass mill. Environ. Pollut Ser A Ecol Biol 1983; 30: 87–108.

42. Roelofs, D., Aarts, M.G.M., Schat, H., van Straalen, N.M. Functional ecological genomics to demonstrate general and specific responses to abiotic stress. Funct Ecol 2008; 22:8-18.
43. Simonsen, V., Laskowski, R., Bayley, M., Holmstrup, M. Low impact of metal pollution on genetic variation in the earthworm *Dendrobaena octaedra* measured by allozymes. Pedobiologia 2008; 52: 51-60.
44. Valko, M., Morris, H., Cronin, M.T.D. Metals, toxicity and oxidative stress. Curr Med Chem 2005; 12:1161–1208.
45. Kozlov, M. V., Zvereva, E. L. A second life for old data: Global patterns in pollution ecology revealed from published observational studies. Environ Pollut 2011; 159:1067-1075.
46. Lavelle, P., Decaens, T., Aubert, M., Barot, S., Blouin, M., Bureau, F., Margerie, P., Mora, P., Rossi, J.-P. Soil invertebrates and ecosystem services. Eur J Soil Biol 2006; 42:S3-S15.
47. Edwards, C.A., Bohlen, P.J. Biology and Ecology of Earthworms, 1996; third ed. Chapman & Hall, London.
48. Spurgeon, D.J., Weeks, J.M., van Gestel, C.A.M. A summary of eleven years progress in earthworm ecotoxicology. Pedobiologia 2003; 47:588-606.
49. Sturzenbaum, S.R., Andre, J., Kille, P., Morgan, A.J. Earthworm genomes, genes and proteins: the (re)discovery of Darwin's worms. Proc R Soc B 2009; 276:789-797.
50. Spurgeon. D. J., Hopkin, S. P. The effects of metal contamination on earthworm populations around a smelting works: quantifying species effects. Appl Soil Ecol 1996; 4: 147-160.
51. Lukkari, T., Taavitsainen, M., Väisänen, A., Haimi, J. Effects of heavy metals on earthworms along contamination gradients in organic rich soils. Ecotox Environ Safe 2004; 59:340-348.
52. Leveque, T., Capowiez, Y., Schreck, E., Mombo, S., Mazzia, C., M., Foucault, Y., A., Dumat, C. Effects of historic metal(loid) pollution on earthworm communities. Sci of Total Environ 2015; 511: 738-746.
53. Ross, K., Cooper, N., Bidwell, J. R., Elder, J. Genetic diversity and metal tolerance of two marine species: a comparison between populations from contaminated and reference sites. Mar Pollut Bull 2002; 44: 671-679.
54. van Straalen, N. M., Timmermans, M. J. T. N. Genetic variation in toxicant-stressed populations: An evaluation of the "genetic erosion" hypothesis. Hum and Ecol Risk Asses 2007;8: 983-1002.
55. Ungherese, G., Mengoni, A., Somigli, S., Baroni, D., Focardi, S., Ugolini, A. Relationship between heavy metals pollution and genetic diversity in Mediterranean populations of the sandhopper Talitrus saltator (Montagu) (Crustacea, Amphipoda). Environ Pollut 2010; 158: 1638-1643.
56. Haimi, J., Knott, K.E., Yliniemi, I. Does metal contamination affect clonal diversity of the parthenogenetic earthworm *Dendrobaena octaedra*? Eur J Soil Biol 2007; 43:268-272.

57. Knott, K.E. & Haimi, J. High mitochondrial DNA sequence diversity in the parthenogenetic earthworm *Dendrobaena octaedra*. Heredity 2010; 105:341–347.
58. Haimi, J., Knott, K. E., Selonen, S., Laurikainen, M. Has long-term metal exposure induced changes in life history traits and genetic diversity of the enchytraeid worm *Cognettia sphagnetorum* (Vejd)? Environ Pollut 2006; 140:463-470.
59. Martins, N., Bollinger, C., Harper, R. M., Ribeiro, R. Effects of acid mine drainage on the genetic diversity and structure of a natural population of *Daphnia longispina*. Aquat Toxicol 2009; 92:104-112.
60. Costa, D., Marien, J., Janssens, T. K. S., van Gestel, C. A. M., Driessen, G., Sousa, J. P., van Straalen, N. M., Roelofs, D. Influence of adaptive evolution of cadmium tolerance on neutral and functional genetic variation in *Orchesella cincta*. Ecotoxicol 2012; 21: 2078-2087.
61. Costa, D., Bouchon, D., van Straalen, N. M., Sousa, J. P., Ribeiro, R. Copper tolerance and genetic diversity of *Porcellionides sexfasciatus* (isopoda) in a highly contaminated mine habitat. Environ Toxicol Chem 2013; 32:884-888.
62. Giska, I., Babik, W., van Gestel, C. A., van Straalen, N. M., Laskowski, R. Genome-wide genetic diversity of rove beetle populations along a metal pollution gradient. Ecotoxicol Environ Saf 2015; 119: 98-105.
63. Sylvain, Z. A., Wall, D. H. Linking soil biodiversity and vegetation: Implications for changing planet. Am J of Botany 2011; 98:517-527.
64. Terhivuo, J., Saura, A. Dispersal and clonal diversity of North-European parthenogenetic earthworms. Biol Invasions 2006; 8:1205-1218.
65. Omodeo, P. Cariologia dei Lumbricidae. II. Contributo. Caryologia 1955; 8:135–178.
66. Hongell, K., Terhivuo, J. Chromosomal status of the parthenogenetic earthworm *Dendrobaena octaedra* (Sav)(Oligochaete, Lumbricidae) in southern Finland. Hereditas 1989; 110(2):179-182.
67. Terhivuo, J., Saura, A. Allozyme variation in parthenogenetic *Dendrobaena octaedra* (Oligochaeta, Lumbricidae) populations of eastern Fennoscandia. Pedobiologia 1990; 34: 113-139.
68. Mustonen, M., Haimi, J., Väisänen, A., Knott, K.E. Metallothionein gene expression differs in earthworm populations with different exposure history. Ecotoxicol 2014; 23:1732-1743.
69. Heckmann, L., Sorensen, P., Krogh, P., Sorensen, J. NORMA-Gene: A simple and robust method for qPCR normalization based on target gene data. BMC Bioinformatics 2011; 12:250.
70. Terhivuo, J. The Finnish Lumbricidae (Oligichaeta) fauna and its formation. Ann Zool Fennici 1988; 25: 229-247.
71. Salemaa, M. Responses of the understory vegetation of boreal forests to heavy metal loading. Dissertation, 2003, University of Helsinki.
72. Lukkari, T., Aatsinki, M., Väisänen, A., Haimi, J. Toxicity of copper and zinc assessed with three different earthworm tests. Appl Soil Ecol 2005; 30: 133-146.

73. Bindesbol, A., Bayley, M., Damgaard, C., Holmsrup, M. Life-history traits and population growth rate in the laboratory of the earthworm *Dendrobaena octaedra* cultured in copper-contaminated soil. Appl Soil Ecol 2007; 35: 46-56.
74. Fisker, K.V., Sørensen, J.G., Damgaard, C., Pedersen, K.L., Holmstrup, M. Genetic adaptation of earthworms to copper pollution: is adaptation associated with fitness costs in *Dendrobaena octaedra*? Ecotoxicol 2011; 20:563–573.
75. Fisker, K.V., Holmstrup, M., Sørensen, J.G. Variation in metallothionein gene expression is associated with adaptation to copper in the earthworm *Dendrobaena octaedra*. Comp Biochem Physiol C 2013; 157:220-226.
76. Pan, R., Zhang, Z., He, R. Earthworm protease. Appl and Environ Soil Scie 2010; Article ID 294258, 13 pages.
77. Schoofs, L., Clynen, E., Salzet, M. Trypsin and chymotrypsin inhibitors in insects and gut leeches. Curr Pharm Des. 2002; 8(7): 483-91.
78. Sethi, A., Slack, J. M., Kovaleva, E. S., Buchman, G. W., Scharf, M. E. Lignin-associated metagene expression in lignocellulose-digesting termite. Insect Biochem Molec Biol 2013; 43: 91-101.
79. Barski, O. A., Tipparaju, S. M., Bhatnagar, A. The Aldo-Keto Reductase Superfamily and its Role in Drug Metabolism and Detoxification. Drug Metab Rev 2008; 40:553-624.
80. Prabha, M. L., Jayaraaj, I. A., Jeyaraaj, R., Rao, S. Comparative studies on the digestive enzymes in the gut of earthworms, *Eudrilus eugenia* and *Eisenia fetida*. Indian J of Biotechn 2007; 6: 567-569.
81. Yang, C.Y., Li, J.P., Chiu, L.L., Lan, J. L., Chen, D. Y., Chuang, H. C., Huang, C. Y., Tan, T. H. Dual-specificity phosphatase 14 (DUSP14/MKP6) negatively regulates TCR signaling by inhibiting TAB1 activation. J Immunol. 2014;15:1547-1557.

Table 1. Experimental setup.

	Uncontaminated			Cu-contaminated		
Low species diversity	LD1 (6) HD1 (4)	LD2 (4) HD2 (4)	LD3 (4) HD3 (4)	LD1 (6) HD1 (4)	LD2 (4) HD2 (4)	LD3 (4) HD3 (4)
High species diversity	LD1 (6) HD1 (4)	LD2 (4) HD2 (4)	LD3 (4) HD3 (4)	LD1 (6) HD1 (4)	LD2 (4) HD2 (4)	LD3 (4) HD3 (4)

LD = low diversity, HD = high diversity of *Dendrobaena octaedra*, number after LD refers to the clone family, number after HD refers to the genotype combination. Number of replicates of each treatment is shown in parentheses. Additional file 1 shows the genotypes included in the LD and HD treatments.

Table 2. Differences in NH_4-N concentration between low diversity (LD) and high diversity (HD) treatments

	Overall		First year		Second year	
	X^2	p	X^2	p	X^2	p
General	0.027	0.871	0.027	0.869	0.057	0.811
Uncontaminated -	2.542	0.111	1.653	0.199	1.306	0.253
Uncontaminated +	0.857	0.355	2.041	0.153	0.082	0.775
Contaminated -	0.447	0.504	1.000	0.317	0.082	0.775
Contaminated +	1.788	0.181	4.592	**0.032***	0.184	0.668

Differences between LD and HD are analyzed overall and with the first and second years separated, in the different microcosm condition analyzed with Kruskal-Wallis test. Df is 1 in all. "General" describes all conditions combined. A minus-sign indicates the condition with low diversity of other decomposer animals, and a plus-sign indicates the condition with high diversity of other decomposer animals.

Table 3. Difference in CO_2 production between low diversity (LD) and high diversity (HD) treatments

	Overall		First year		Second year	
	X^2	p	X^2	p	X^2	p
General	3.904	**0.048***	5.098	**0.024***	0.009	0.925
Uncontaminated -	0.565	0.452	2.677	0.102	0.919	0.338
Uncontaminated +	2.759	0.097	7.181	**0.007****	2.255	0.133
Contaminated -	3.453	0.063	3.495	0.062	0.697	0.404
Contaminated +	1.359	0.244	3.392	0.066	0.633	0.426

Differences between LD and HD are analyzed overall and separately for the first year and second year. Kruskal-Wallis test was used with df = 1. Conditions labelled as in Table 2.

Table 4. Differences in earthworm biomass between low diversity (LD) and high diversity (HD) treatments.

	First year		Second year	
	F	p	F	p
General	0.176	0.675	7.869	**0.006****
Uncontaminated -	0.002	0.966	1.316	0.257
Uncontaminated +	0.054	0.818	0.920	0.346
Contaminated -	0.490	0.486	0.108	0.744
Contaminated +	0.124	0.726	13.520	**0.001*****

Differences between LD and HD are analyzed separately for the first year and the second year with one-way ANOVA, with biomass at the start as a covariant and df = 1. Conditions labelled as in Table 2.

Table 5. Differences in gene expression between low diversity (LD) and high diversity (HD) treatments

		Overall		First year		Second year	
Comparison	Gene	F	p	F	p	F	p
General	AkRed	1.887	0.171	0.035	0.851	4.069	**0.046***
	ChymInh	6.057	**0.014***	4.557	**0.035***	1.665	0.199
	DualPhos	0.045	0.832	0.862	0.355	0.373	0.543
	GlucReg	0.801	0.372	0.365	0.547	0.481	0.489
	PanAmy	0.710	0.400	0.004	0.951	1.164	0.283
	MT	0.109	0.741	0.236	0.628	0.964	0.328
Uncontaminated-	AkRed	0.991	0.323	3.560	0.068	0.016	0.899
	ChymInh	14.22	**<0.001*****	8.577	**0.006****	6.097	**0.019***
	DualPhos	0.672	0.415	0.806	0.375	0.114	0.737
	GlucReg	1.593	0.211	0.095	0.760	2.194	0.147
	PanAmy	0.055	0.815	3.720	0.062	1.300	0.262
	MT	1.581	0.213	2.335	0.135	0.102	0.751
Uncontaminated+	AkRed	5.103	**0.028***	2.219	0.148	2.164	0.153
	ChymInh	2.279	0.137	2.079	0.161	0.006	0.939
	DualPhos	5.545	**0.022***	0.907	0.349	4.906	**0.035***
	GlucReg	0.242	0.625	0.453	0.506	0.537	0.470
	PanAmy	0.377	0.542	2.787	0.106	0.187	0.669
	MT	6.226	**0.015***	8.994	**0.006****	0.320	0.576
Contaminated-	AkRed	3.517	0.065	0.281	0.600	5.961	**0.021***
	ChymInh	0.979	0.326	4.304	**0.045***	0.976	0.331
	DualPhos	0.352	0.555	2.174	0.149	9.209	**0.005****
	GlucReg	0.052	0.820	0.168	0.685	0.218	0.644
	PanAmy	0.366	0.547	0.069	0.795	1.712	0.201
	MT	0.785	0.357	3.020	0.091	0.708	0.407
Contaminated+	AkRed	0.287	0.594	0.142	0.709	0.095	0.760
	ChymInh	1.078	0.303	0.991	0.328	0.850	0.364
	DualPhos	0.155	0.696	0.017	0.896	0.149	0.702
	GlucReg	2.139	0.149	3.831	0.060	0.313	0.580
	PanAmy	0.889	0.350	0.690	0.413	0.226	0.638
	MT	0.658	0.862	0.077	0.784	0.977	0.331

Differences between LD and HD are analyzed overall and for the first and second years separately with one-way ANOVA with df = 1. Conditions labelled as in Table 2.

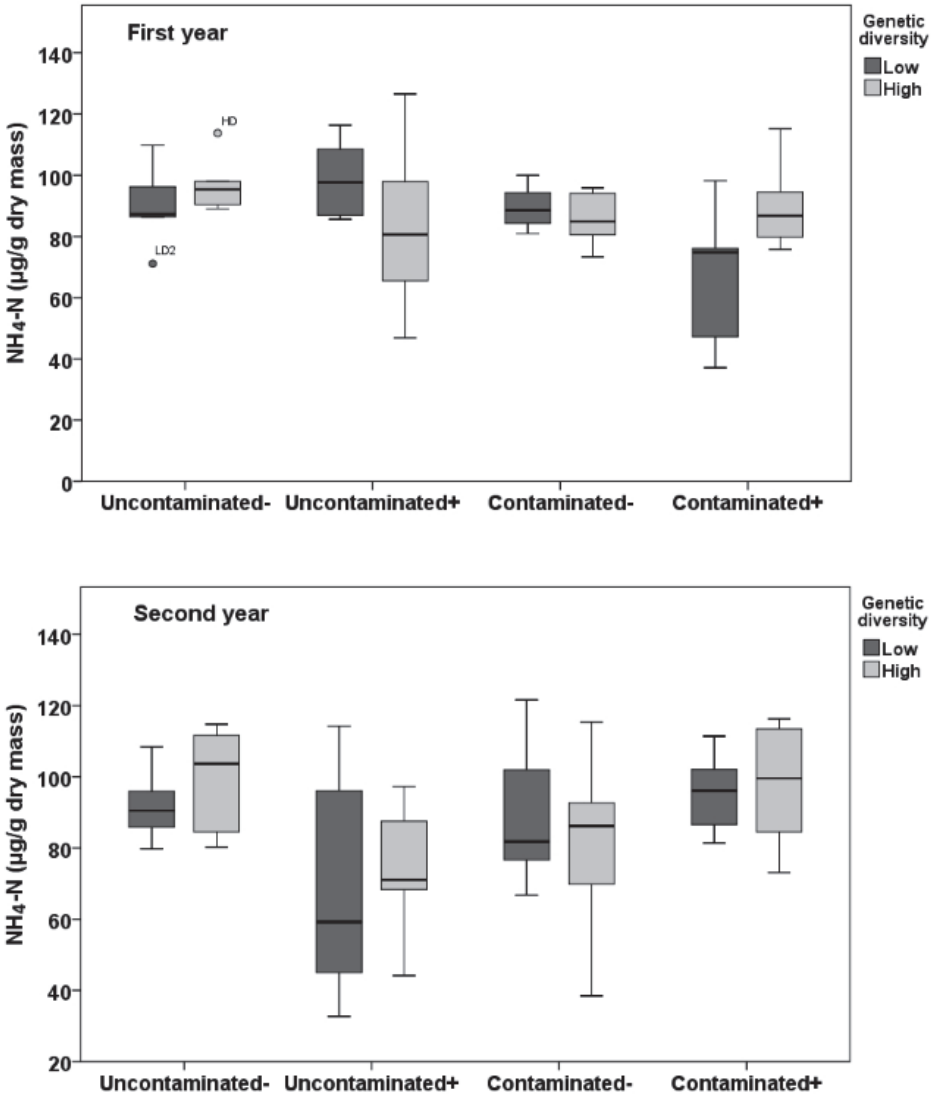


Figure 1. Concentration of NH_4-N (µg/g dry mass) in low diversity (LD; dark grey) and high diversity (HD; light grey) treatments exposed to different conditions (uncontaminated or Cu-contaminated soil, low or high diversity of the other decomposer animals, indicated by the – and + signs, respectively). Data from the first and second years of the experiment are shown separately.

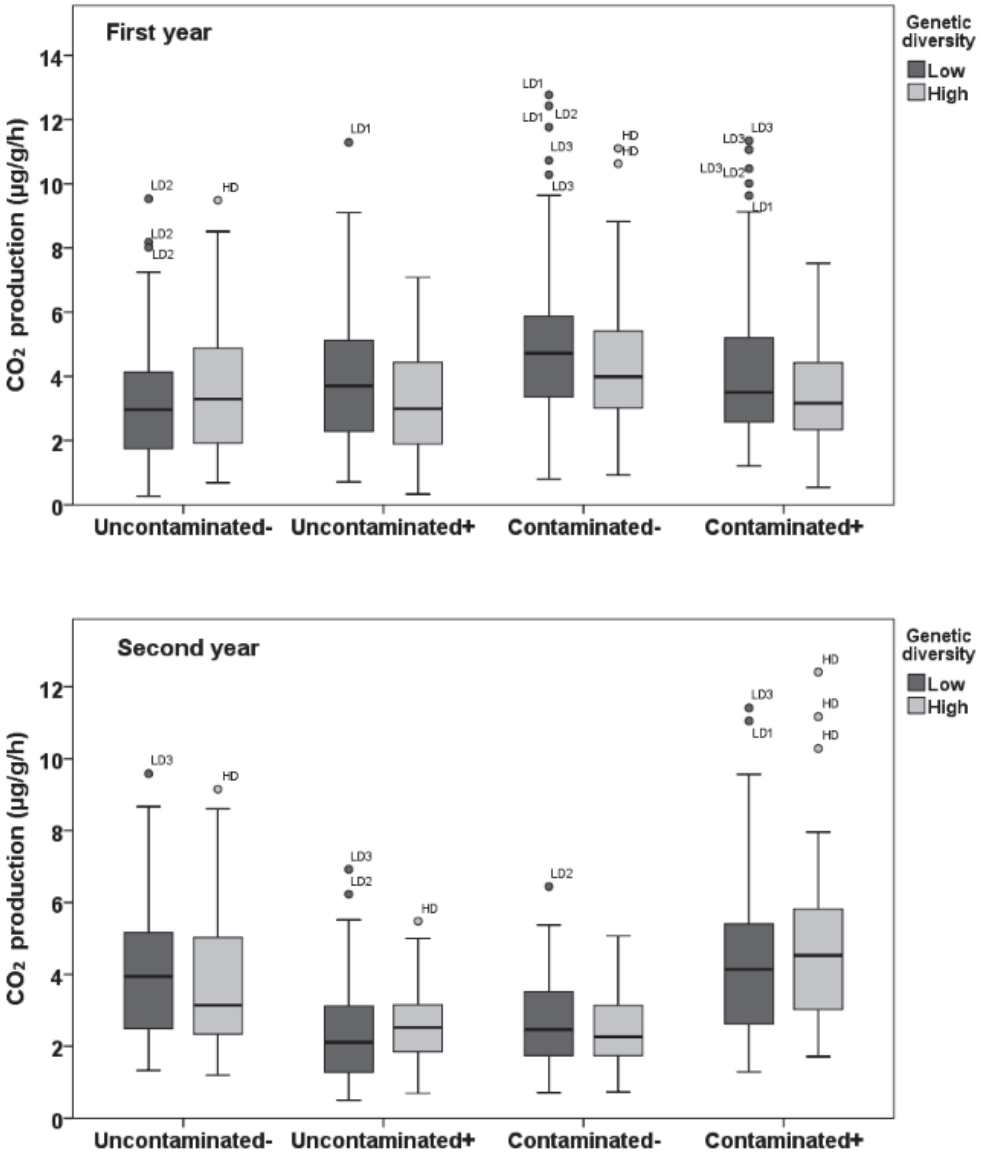


Figure 2. CO_2 production (µg/g/h; means of all measurements done throughout the experiment) in low diversity (LD; dark grey) and high diversity (HD; light grey) treatments exposed to different conditions (uncontaminated or Cu-contaminated soil, low or high diversity of other decomposer animals). Data from the first and second years of the experiment are shown separately.

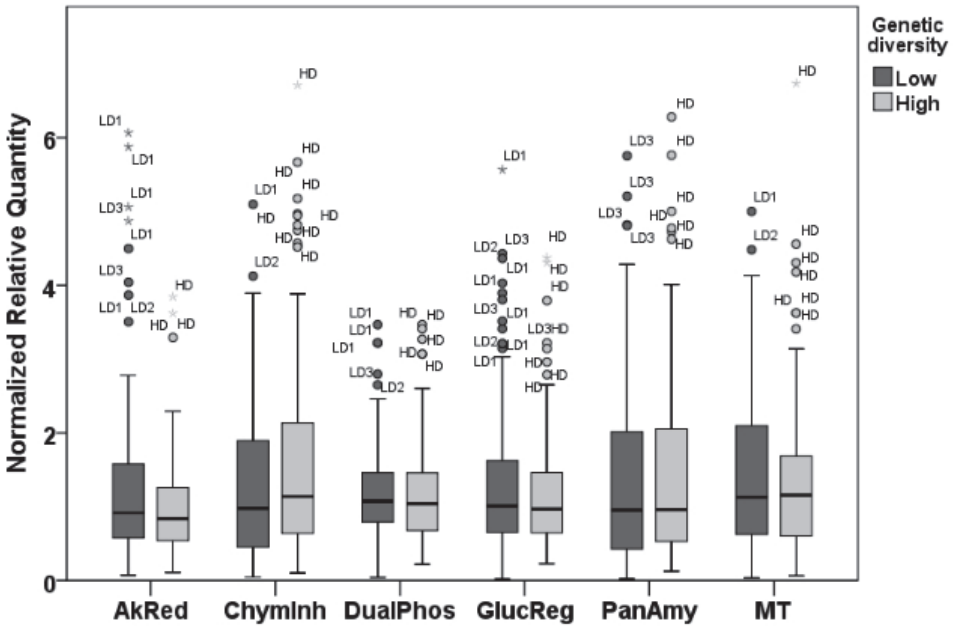


Figure 3. Gene expression (Normalized Relative Quantity) in the low diversity (LD; dark grey) and high diversity (HD; light grey) treatments overall (both years and all conditions combined).

SUPPLEMENTARY DATA

Additional file 1. Genotypes of the earthworms used in the microcosm-experiment. In each microcosm of LD1 there were four earthworms from clone family H1 (three in the treatment with high diversity of other decomposer animals). Likewise, LD2 contained four (or three) earthworms from clone family H2, and LD3 contained four (or three) earthworms from clone family H3. Clone families H2 and H3 are the same genotype based on the five microsatellite markers used here (DO1, DO2, DO3, DO4, DO6). High diversity microcosms contained earthworms with differing genotypes. In high diversity of other decomposer animals, the genotype of the worm replaced by *Dendrodrilus rubidus* is marked in parentheses. Each microcosm of HD1 was composed of H1/H4/H8/(H6), each microcosm of HD2 was composed of H1/H8/H9/(H5), and each microcosm of HD3 was composed of H1/H2/H7/(H10).

	DO1	DO2	DO3	DO4	DO6
H1	231/237	140/153	165/173/177	211/213	96/100
H2	231	140	173/181/189	211/213	96
H3	231	140	173/181/189	211/213	96
H4	231/237	138/140	165/177/185/200	211/213	90/96
H5	229/231/233/235/237	131/138/143/145	165/167/171/177	211/213	90/96/100
H6	229/231/233/235/237	138/145/159	165/177/181/187/196	211/213	96/100
H7	227/229/231/233/235/237	138/145	165/173/177	211/213	100
H8	227/229/231/233/235/241	140	165/177/185	211/213	96
H9	229/231/233/241/245/253	140/147/151	177/185/187/193	211/213	90/96
H10	229/231/233/235/237	138/145	165/173/177	211/213	96/100

DNA extraction and genotyping

Genomic DNA was extracted from piece of the anterior end of the earthworm using Qiagen chemistry (DNeasy kit reagents) and a Kingfisher magnetic processor (Thermo Fisher Scientific). Five microsatellite loci were used for genotyping, DO1, DO2, DO3, DO4 and DO6 (described in Mustonen et al. unpublished). Amplification was performed separately for each locus in 10 µl reactions containing 1 µl of template DNA, 1X buffer (Biotools), 0.4 mM dNTPs (Fermentas), 1 µM reverse primer, 0.9 µM forward primer (TAG Copenhagen), 0.1 µM labeled forward primer (Applied Biosystems), 2 mM MgCl2 (Biotools) and 0.5 units Taq polymerase (Biotools). Conditions for thermocycling were 94 °C for 3 min, then 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, ending with a final extension of 72 °C for 10 min. Amplification products were separated using the ABI PRISM 3130xl with GeneScan 500 LIZ size standard and genotyped using GeneMapper 5 software (all Applied Biosystems).

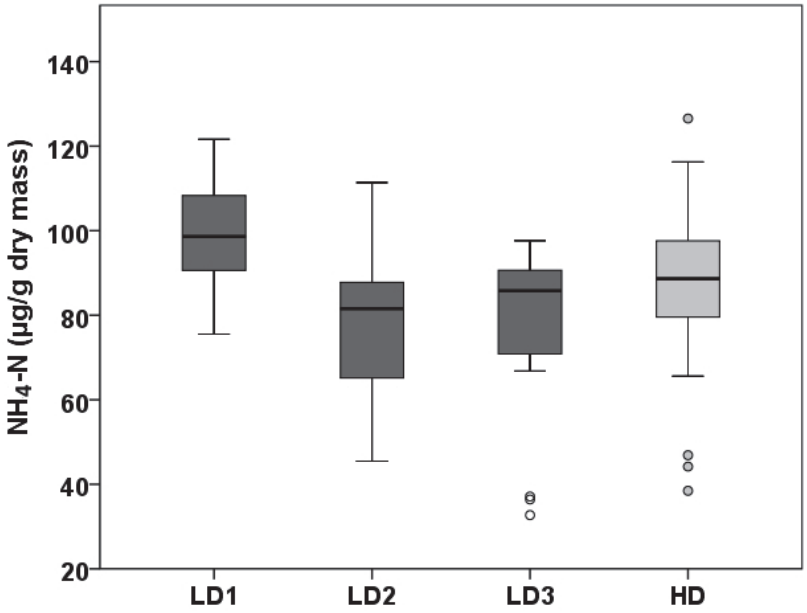
Additional file 2. Primer sequences used in qPCR and reaction efficiencies.

Gene	Primer (5'-3')	Efficiency %
MT-2	F: ACACTCAGTGCTGTGGCAGCG	103,5
	R: GGCTGCGCACTTGCAGGC	
AkRed	F: GCTGATCGAGGCAGATCCTT	96,2
	R: CCAAGTCCACGAACCCTGAA	
ChymInh	F: TCAGCTACCCGGTTCTAGGT	94,8
	R: AAGAGGCCAAAGCACAGGTT	
DualPhos	F: CGTCCAGTCATCCGTCCAAA	100,2
	R: ATAGCTCCAATGGGTGACGG	
GlucReg	F: GACGAGCTCATCAGTTGCAG	94,6
	R: TCTTTCAGGCGGAGGTTGAT	
PanAmy	F: ATCCTGACCTTCTTCGAGGC	97,9
	R: TCCTCGGCCAATAGTAGCTG	
18S	F: ACCACATCCAAGGAAGGCAG	93,3
	R: CCCGAGATCCAACTACGAGC	
28S	F: TGGTGGAGGTCCGCAGCGAT	90,5
	R: CGTTTCGTCCCCAAGGCCTC	

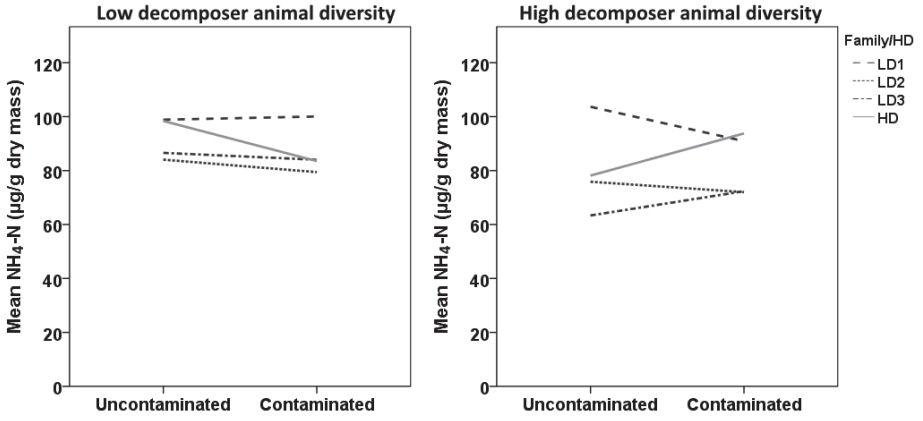
Additional file 3. Comparisons between the different low diversity (LD) clone families, between high diversity (HD) and the two low diversity genotypes separately (LD1 is one genotype, and LD2&3 are another), between uncontaminated and contaminated conditions, and between low and high diversity of other decomposer animals. Analyses were done either with Kruskal-Wallis test (X^2) or one-way ANOVA (F): for nutrient mineralization (X^2), CO_2 production (X^2), cocoon production (X^2), mortality (X^2) and gene expression (F).

	Comparison between									
	LD families		HD vs. LD1		HD vs. LD2&3		Uncont. vs contaminated		Low vs high animal diversity	
	X^2/F	p	X^2/F	p	X^2/F	p	X^2/F	p	X^2/F	p
Nutrients	17.48	**<0.001**	5.467	**0.019**	5.118	**0.024**	0.166	0.683	1.833	0.176
CO_2	2.876	0.237	1.131	0.252	4.148	**0.042**	29.65	**<0.001**	0.284	0.594
Cocoons	12.63	**0.002**	9.460	**0.002**	0.626	0.429	0.094	0.759	3.017	0.082
Mortality	0.815	0.665	1.076	0.300	2.898	0.089	0.267	0.605	0.597	0.440
Gene Exp.										
AkRed	12.50	**<0.001**	19.29	**<0.001**	1.998	0.159	3.190	0.075	0.009	0.923
ChymInh	1.174	0.312	9.231	**0.003**	1.809	0.180	0.783	0.377	9.589	**0.002**
DualPhos	1.770	0.174	0.757	0.385	0.145	0.704	29.66	**<0.001**	0.002	0.965
GlucReg	1.898	0.154	2.415	0.122	0.022	0.883	0.019	0.890	1.456	0.229
PanAmy	5.920	**0.003**	6.785	**0.010**	0.785	0.377	0.009	0.924	0.527	0.469
MT	0.656	0.520	0.389	0.534	<0.001	0.996	8.572	**0.004**	6.750	**0.010**

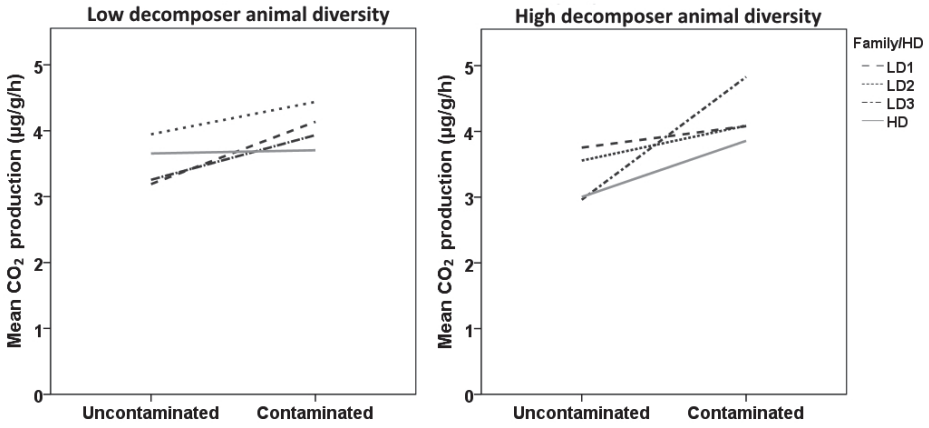
Additional file 4. Concentrations of NH_4-H in the presence of the different low diversity families (LD1, LD2 and LD3) and the high diversity treatment (HD).



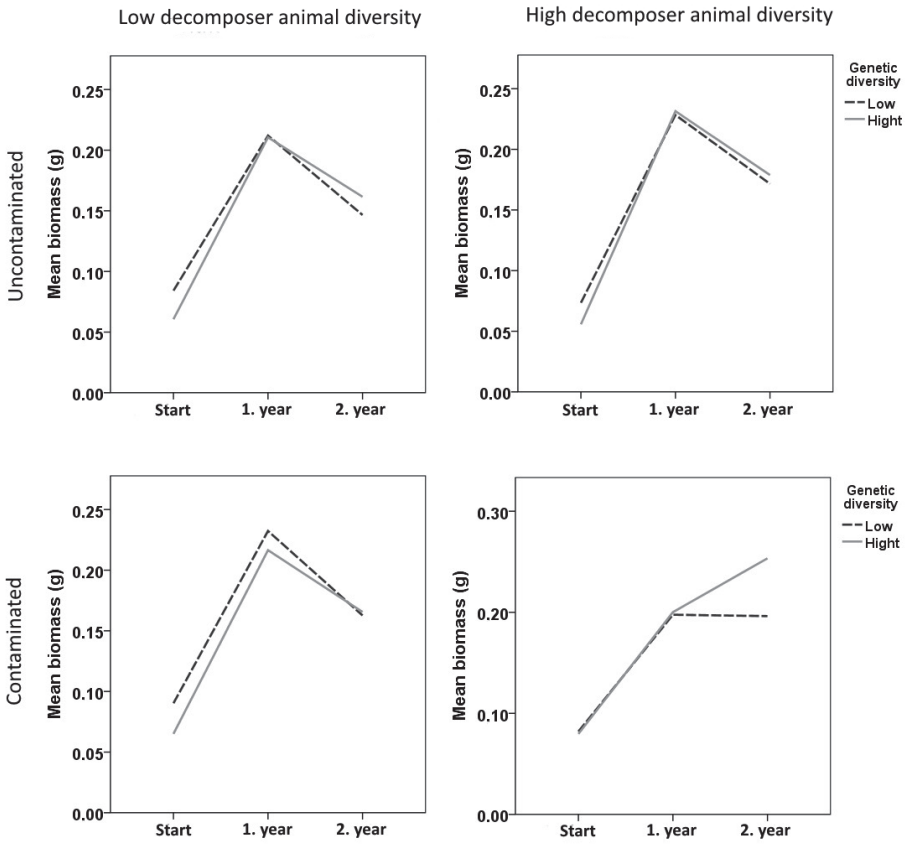
Additional file 5. Overall nutrient mineralization (concentration of NH_4-H) response of the different low diversity families (LD1, LD2 and LD3) and the high diversity treatment (HD) to different conditions (uncontaminated or contaminated, both in low and high diversity of other decomposer animals).



Additional file 6. Overall CO_2 production response of the different low diversity families (LD1, LD2 and LD3) and t the high diversity treatment (HD) to different conditions (uncontaminated or contaminated, both in low and high diversity of other decomposer animals).



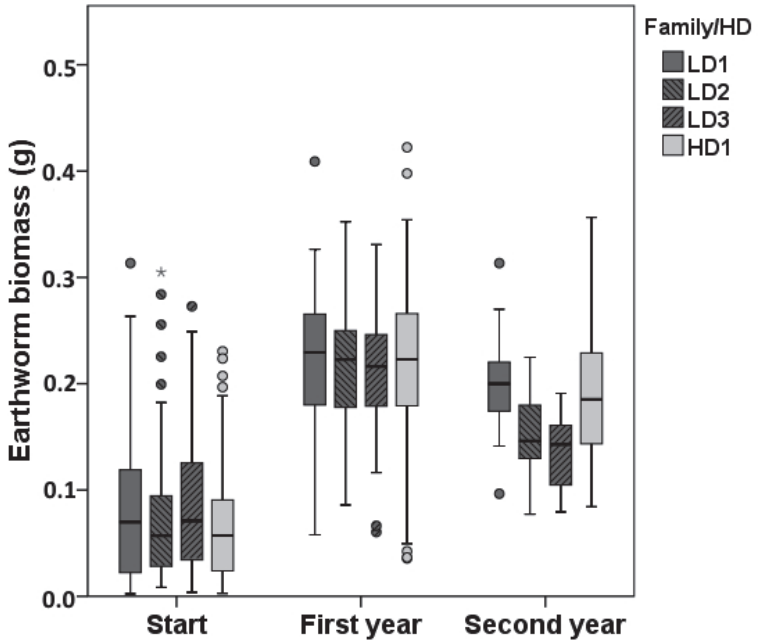
Additional file 7. Biomass (g) of the earthworms in low diversity and high diversity treatments in the start of the experiment, after the first year and after the second year in different conditions (uncontaminated or uncontaminated, low or high diversity of other decomposer animals).



Additional file 8. Comparisons of the biomass of *D. octaedra* between the different low diversity (LD) clone families, between high diversity (HD) and the two low diversity genotypes separately (LD1 is one genotype, and LD2&3 are another), between uncontaminated and contaminated conditions, and between low and high diversity of other decomposer animals for the first and second year separately. Analyses were done with one-way ANOVA, with starting weight as a covariate.

	Comparison between									
	LD families		HD vs. LD1		HD vs. LD2&3		Uncont. vs contaminated		Low vs high animal diversity	
	F	p	F	p	F	p	F	p	F	p
1. year	0.673	0.511	0.841	0.360	0.016	0.900	0.534	0.465	0.210	0.647
2. year	23.427	**<0.001**	0.237	0.627	23.010	**<0.001**	11.191	**0.001**	27.362	**<0.001**

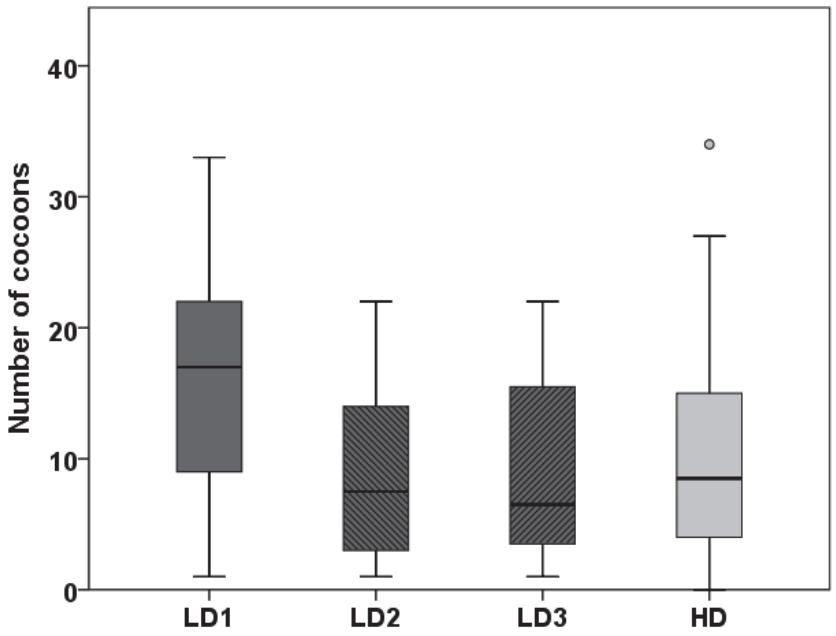
Additional file 9. Biomass (g) of the earthworms in the different low diversity families (LD1, LD2 and LD3) and the high diversity treatment (HD) at the start of the experiment, after the first year and after the second year.



Additional file 10. Differences in cocoon production between low diversity (LD) and high diversity (HD) treatments overall and when the first year and second year were analyzed separately for the different microcosm conditions (uncontaminated or contaminated soil, low or high diversity of other decomposer animals). For all, Kruskal-Wallis tests with df = 1. "General" describes all conditions combined. A minus-sign indicates the condition with low diversity of other decomposer animals, and a plus-sign indicates the condition with high diversity of other decomposer animals

	Overall		First year		Second year	
	X^2	p	X^2	p	X^2	p
General	1.330	0.249	0.642	0.423	0.403	0.526
Uncontaminated -	0.672	0.412	0.096	0.756	0.021	0.886
Uncontaminated +	0.191	0.662	0.417	0.519	0.185	0.667
Contaminated -	1.651	0.199	2.244	0.134	0.083	0.774
Contaminated +	0.125	0.724	0.732	0.392	0.021	0.886

Additional file 11. Number of cocoons produced by different low diversity families (LD1, LD2 and LD3) and high diversity treatment (HD).



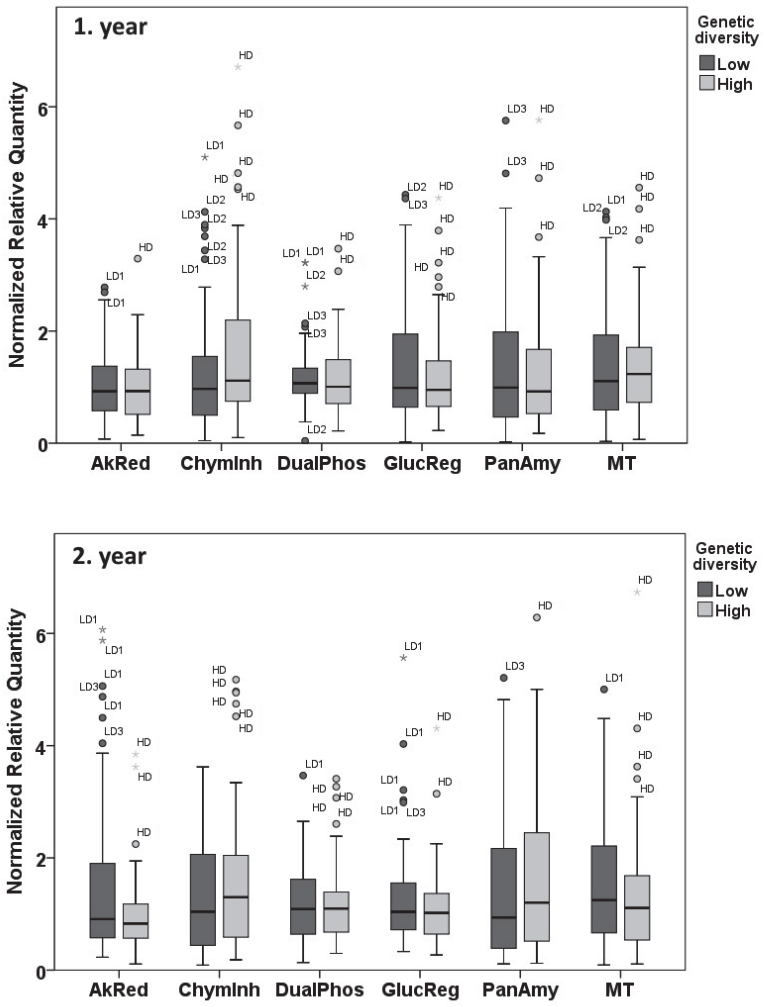
Additional file 12. Differences in mortality between low diversity (LD) and high diversity (HD) treatments overall and when the first year and second year were analyzed separately for the different microcosm conditions (uncontaminated or contaminated soil, low or high diversity of other decomposer animals). For all, Kruskal-Wallis tests with df = 1. "General" describes all conditions combined. A minus-sign indicates the condition with low diversity of other decomposer animals, and a plus-sign indicates the condition with high diversity of other decomposer animals. Also listed is the number of earthworms that died in each case.

	Overall		First year		Mortality		Second year		Mortality	
	X^2	p	X^2	p	LD	HD	X^2	p	LD	HD
General	2.654	0.103	4.278	**0.029**	11	19	1.219	0.270	14	17
Uncontaminated -	3.426	0.064	4.172	**0.041**	1	5	1.167	0.280	0	4
Uncontaminated +	0.122	0.717	1.583	0.208	2	6	1.221	0.269	7	2
Contaminated -	2.439	0.118	0.448	0.504	3	4	2.164	0.141	4	8
Contaminated +	0.128	0.721	0.031	0.861	5	4	0.336	0.562	3	3

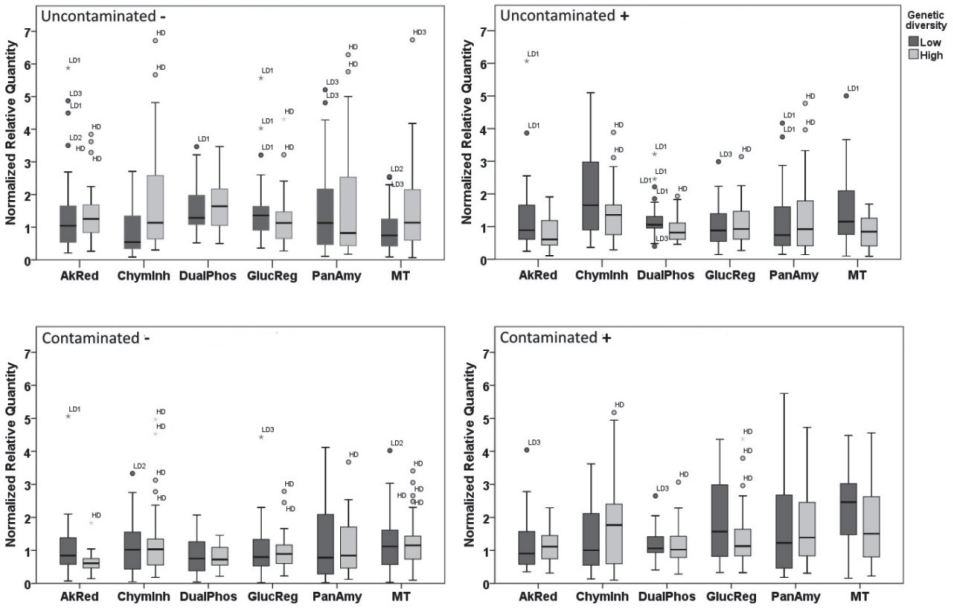
Additional file 13. Amount of each of the other species in each of the condition: uncontaminated or contaminated soil, low (marked with minus-sign) or high diversity of other decomposer animals (marked with +-sign), both after the first year and the second year.

	Uncontaminated -		Uncontaminated +		Contaminated -		Contaminated +	
	1.year	2.year	1.year	2.year	1.year	2.year	1.year	2.year
Nematodes	1156	393	679	1313	742	368	1021	1064
Enchydraeids	0	0	951	51	1	14	620	263
Collembola	77	0	20	5	33	5	134	218
Mesostigmata	3	0	37	21	1	0	36	67
Oribatida	34	5	121	33	40	4	124	156

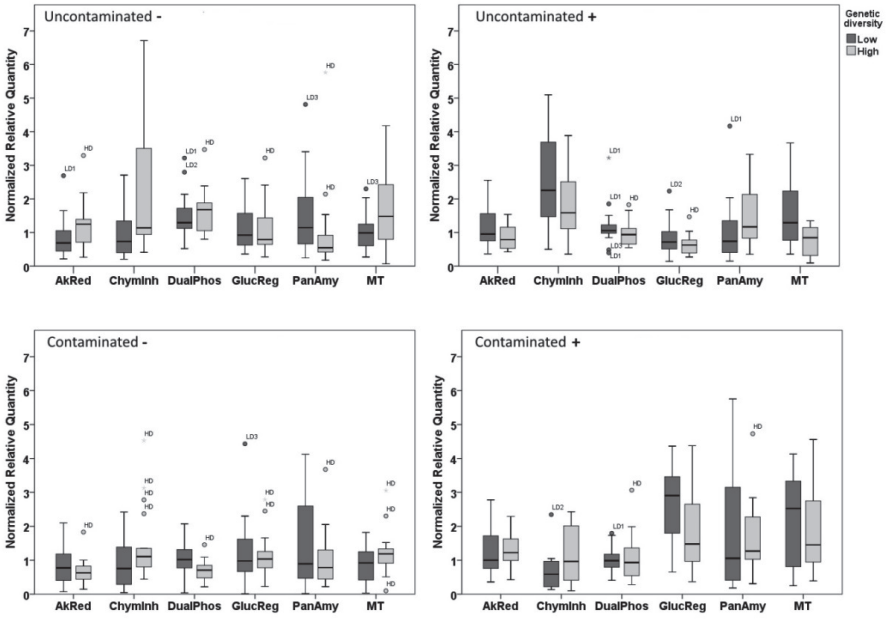
Additional file 14. Gene expression (Normalized Relative Quantity) in the low diversity and high diversity treatments in general (conditions combined) for the first year and second year separately.



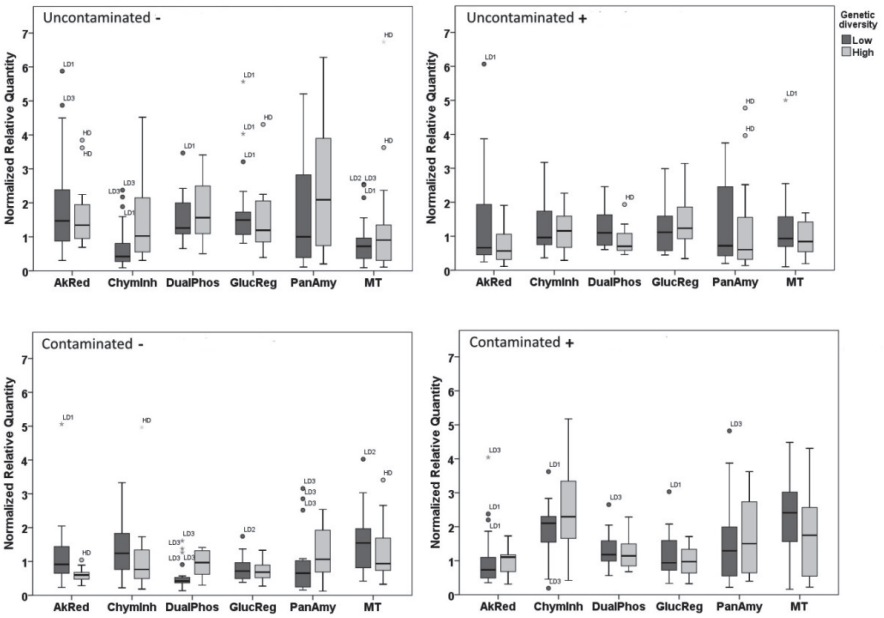
Additional file 15. Gene expression (Normalized Relative Quantity) in the low diversity and high diversity treatments for the different conditions: uncontaminated or contaminated, low (marked with minus-sign) or high diversity of other decomposer animals (marked with +-sign) overall of the experiment (first year and second year are not separated).



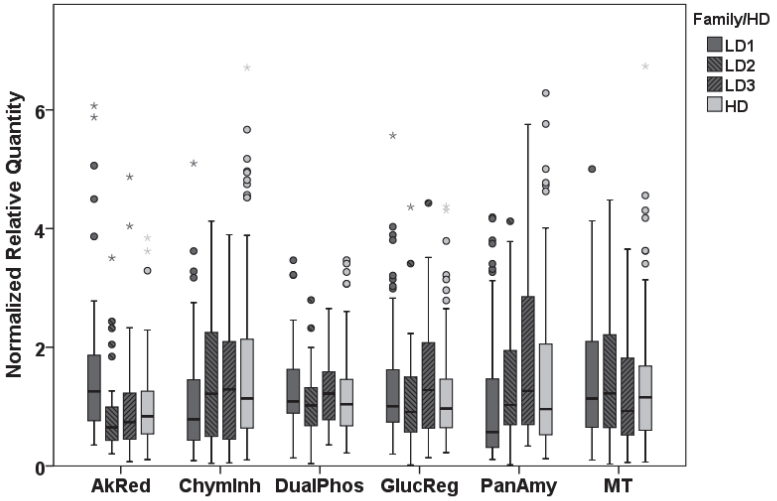
Additional file 16. Gene expression (Normalized Relative Quantity) in the low diversity and high diversity treatments for the different conditions: uncontaminated or contaminated, low (marked with minus-sign) or high diversity of other decomposer animals (marked with +-sign) for the first year.



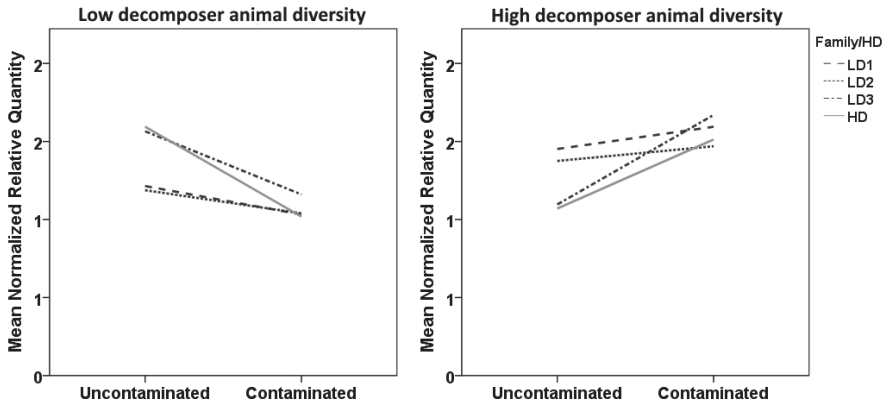
Additional file 17. Gene expression (Normalized Relative Quantity) in the low diversity and high diversity treatments for the different conditions: uncontaminated or contaminated, low (marked with minus-sign) or high diversity of other decomposer animals (marked with +-sign) for the second year.



Additional file 18. Gene expression (Normalized Relative Quantity) in the different low diversity families (LD1, LD2 and LD3) and high diversity treatment (HD).



Additional file 19. Overall (all genes combined) gene expression response of the different low diversity families (LD1, LD2 and LD3) and the high diversity treatment (HD) to different conditions (uncontaminated or contaminated, both in low and high diversity of other decomposer animals).



IV

GOING DIGITAL - A NEW TECHNIQUE FOR QUANTIFYING GENE EXPRESSION IN MOLECULAR ECOLOGY

by

Marina Mustonen & K. Emily Knott

Manuscript