



## Review

# Using biomarkers in an evolutionary context: Lessons from the analysis of biological responses of oligochaete annelids to metal exposure



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## ABSTRACT

Anthropogenic activities may lead to the accumulation of inorganic and organic compounds in topsoils. Biota living in close contact with contaminated soils may experience stress at different levels of biological organization throughout the continuum from molecular to community level. Biological responses observed at the individual or infra-individual level of biological organization led to the development of biomarkers. The development of biomarkers consists often in evidencing biological modifications following a contaminant stress in laboratory conditions, using na ive organisms and it is sometime proposed to use the biological state of individuals from sentinel species collected in the field to evaluate the level of environmental exposure. However, considering the possibility of local adaptation following long-term exposure, organisms response sampled in the field may substantially differ from laboratory specimens. In this review, we discuss this point focusing on the definition and validity of molecular biomarkers of metal pollution using earthworms of the Lumbricidae family.

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## 1. The concept of biomarker in ecotoxicology: definition and limits

Ecotoxicology is a discipline at the interface between ecology and toxicology. Concisely, ecotoxicology is concerned with toxic molecules, hereafter called contaminants, either of artificial origin (including drugs, endocrine disruptors...) or natural agents (metals, arsenic,...), of which Human beings alter the distribution and/or cycle in the different compartments of the biosphere. From a fundamental point of view, ecotoxicology studies the fate and consequences of contaminants on biological systems, from ecosystems to individual organisms. From an applied point of view, it is increasingly asked to ecotoxicologists to develop tools allowing to determine the intensity and duration of contamination events and to assess associated ecological risks, through the prediction of potential effects of contaminant exposure in nature.

One approach to meet this social demand for biomonitoring methods is the development of biomarkers. This approach considers that the best method to detect the biological impact of

contaminant exposure is to investigate the effects of contaminants on biological systems. Indeed, compared to traditional methods focusing on physical and chemical properties of soils, biomarkers are assumed to focus on the effects of the bioavailable (*i.e.* transmitted to living organisms) fraction of environmental chemicals and to integrate the putative interactive effects of complex mixtures of chemicals in the Ecological Risk Assessment (ERA).

Theoretically, a "biomarker" can be defined from any observable and/or measurable functional response to exposure to one or several contaminants that can be characterized at the sub-individual level of biological organization (molecular, biochemical, cellular, physiological, behavioral). Importantly, the response is assumed to indicate a departure from healthy status that cannot be detected from an intact organism (Weeks, 1995; van Gestel and van Brummelen, 1996; Ricketts et al., 2004). The concept of biomarker is thus based on the causal relationship between the contamination of environments by any chemical inducing a stress (pesticides, polycyclic aromatic hydrocarbons (PAHs), metals,...) and biological changes induced by the contaminated environment.

In practice, the development of biomarkers can be divided in two, reciprocally fundamental and applied, steps. First, it consists in characterizing in laboratory conditions the effects of contaminant exposure on the biology of an organism of interest. Second, it

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consists in using the characterized response to assess the level and duration of contaminant exposure of organisms sampled in the field (Weeks, 1995).

### 1.1. Fundamental approaches

Characterizing the effects of contaminant exposure on the biology of an organism is mostly a fundamental issue based on experimental approaches. It requires accumulating fundamental knowledge in the hazardous nature of a substance which is evaluated by toxicity studies (acute or chronic, intrinsic or cocktails...) and establishing thresholds beyond which a substance has a toxic effect or below which it is harmless. These thresholds usually rely on the physical and chemical properties of the contaminant, the characteristics of the environment, the duration of exposure (chronic, occasional), the route of exposure (percutaneous, ingestion, inhalation...) and the state of development of exposed individual (species, sex, age...). In experimental conditions, the comparison of phenotypes of conspecific individuals differentially exposed to one or several contaminants over short periods of time (less than a generation) is supposed to reveal the biological response to contaminant(s). In recent years, these ecotoxicological investigations have largely benefited from the emergence of molecular biology techniques, which led to a better understanding of the mechanisms of contaminants action at a molecular level. In particular, the advent of transcriptomic tools (real time PCR, subtractive libraries, pyrosequencing of cDNA libraries, DNA chips) coupled to bioinformatic procedures and statistical data analysis, allowed a comprehensive analysis of gene expression in various ecotoxicological contexts (Brulle et al., 2010). The main advantage of gene expression studies is that gene expression profiles represent the first level of integration between environmental stressors and the genome which, through the synthesis of proteins, determines the response of the organisms to external changes (Brulle et al., 2008; Brulle et al., 2010).

Interestingly, model organisms used in fundamental ecotoxicological studies are often "naive", which means that they belong to model species and/or test species that have never been previously exposed to a contaminant and are not descended from exposed individuals. In such cases, it seems reasonable to assume that phenotypic variation observed among individuals in contaminated and control conditions reveals environmentally induced modifications and may therefore not be accounted for by genetic differences among individuals. The ability to adjust phenotypes in response to environmental changes is known as "phenotypic plasticity" (Pigliucci, 2005; Van Kleunen and Fischer, 2005; Ghalambor et al., 2007) in evolutionary biology, mostly called "acclimation" in ecotoxicology. This phenomenon is a key concept in the definition of biomarkers that are commonly considered as indicators for environmentally induced responses.

### 1.2. Applied ecotoxicology

In contrast to fundamental approaches exposed above, the application of biomarkers for ERA purposes relies on more technical issues. Two main conceptual differences can be highlighted when one shifts from the experimental accumulation of fundamental knowledge about biological responses to contaminant exposure in laboratory conditions to the practical development of biomarkers that have to be usable in the field.

- Firstly, biomarkers should be used on sentinel species, *i.e.* on wild organisms sampled in natural populations from the field rather than on laboratory specimens (Van Der Schalie et al., 1999; Beeby, 2001). Working on sentinel species implies that biomarkers may be developed on varying species corresponding

to the ecosystem of interest. Considering the ERA of soil pollution in terrestrial ecosystems, it is well admitted that, because they represent important ecological functions of terrestrial ecosystems, species from the soil macrofauna should be considered as potential indicators of soil quality (Stork and Eggleton, 1992; Van Straalen, 1998; Marhan and Scheu, 2005). A literature survey distinguished a few species belonging to three taxa living in close contact with the soil: nematodes (*Caenorhabditis elegans*), Collembola and oligochaete annelids (Brulle et al., 2010). Since the pioneer observations of Darwin (1883), the key-role of earthworms in soil ecosystems is well-known. In particular, in accordance with their status of soil ecosystem engineers cited in Lavelle and Spain (2001), earthworms from the Lumbricidae family are good candidates for biomonitoring (Rombke et al., 2005; Sanchez-Hernandez, 2006). Indeed, earthworms play an important role in water, nutrients and carbon cycles in terrestrial ecosystems and increase soil fertility and earthworm populations can provide information on soil structure, microclimatic conditions, nutritional status and presence of toxic elements in soils (Christensen, 1988; Edwards and Bohlen, 1996; Edwards, 1998; Kautenburger, 2006). Therefore, they have been adopted by the international community as sentinel species for the study of the potential environmental impact (ERA) of anthropogenic contaminants such as pesticides, hydrocarbons and Metal trace elements (MTEs) (Edwards and Bohlen, 1996; Edwards, 1998; Pearce et al., 2002; Spurgeon et al., 2003; Seeber et al., 2005; Kautenburger, 2006).

- Secondly, using biomarkers in the field generally assumes that the relationship between the contamination of environments and the biological status of an organism is always deterministic. This means that the level of contaminant exposure in the environment could be deduced from consecutive phenotypic values of field-sampled organisms. This assumption suggests that the main source of phenotypic variation is environmental. This however, may be controversial, in particular if characterized individuals have been sampled in natural populations exposed to contaminants over several generations. Indeed, in comparison to short-term exposure of experimental approaches, the long-lasting exposure to contaminant may not only provoke environmental phenotypic variation but also adaptive evolution (Hendry et al., 2011). In particular, when conspecific populations are differentially exposed to metal stress for a relatively long period of time (several generations), among-population phenotypic differences, potentially outmatching differences induced by the local environmental heterogeneity, may be genetically determined (*i.e.* heritable). Thus, indeed, Darwinian selection may have promoted divergent evolution among populations, a phenomenon known as local adaptation (Kawecki and Ebert, 2004), and resulting in differentiated molecular mechanisms underlying tolerance to metal stress.

In this context, the aims of the present work are (1) to report a literature review of knowledge about the general effects of MTEs on earthworms as main sentinel species, (2) to particularly highlight the consequences of a long-term exposure to MTEs on earthworm biology, and (3) to debate the definition and the validity of biomarkers of MTE exposure considering that sentinel species may have evolved under such exposure.

## 2. Biological response of earthworms to MTEs exposure in controlled conditions

Among contaminants, MTEs, are of major environmental and human health concern. Indeed, metals are released in large quantities by human activities (agricultural use of fertilizers, residues

from metalliferous mining and smelting industries, etc., (Hopkin, 1989; Bradl, 2005)) and can reach very high concentrations in soils. Unlike organic pollutants, metals remain in the environment. Consequently, they can accumulate in food chains through different trophic levels, causing toxicity to living organisms, including humans (Nawrot et al., 2006; Peralta-Videoa et al., 2009).

Overall, MTEs soil pollution causes environmental stress leading to dysfunction of ecosystems. MTEs pollution usually reduces both species diversity and species richness (Kozlov and Zvereva, 2011). For example, high levels of MTEs affect soil microbial species causing a decrease in population size (Müller et al., 2001) or a reduction in the species richness of soil bacterial communities (Moffett et al., 2003). Studies focusing on earthworms (Annelida Oligochaeta) have also shown a decrease in population densities (Pizl and Josens, 1995) and changes in community structure (Lukkari et al., 2004) in response to metal pollution. In general, loss of species richness can result in the loss of important ecosystem functions. MTE pollution is for example commonly associated with an accumulation of litter due to a reduction of the mineralization that leads to high organic matter content observed in some heavily polluted sites (Cotrufo et al., 1995). At individual and infra individual levels, many ecotoxicological studies conducted in plants and animals living in close contact with contaminated soil led to a better understanding of the biological consequences (MTEs contents in tissues, enzymes activities, genes expression levels) of the exposure of organisms to toxic levels of MTEs. For example, in plants, MTE exposure can cause a significant decrease of the growth rate of trees (Dickinson et al., 1992). Photosynthesis may also be affected through the hydrolysis of chlorophyll (Manios et al., 2003).

Experiments carried out under controlled conditions on a limited number of oligochaetes annelids species (*Eisenia fetida*, *Eisenia andrei*, *Lumbricus rubellus* mainly) show that exposure to MTEs may affect their physiology (see Spurgeon et al., 2003). For example, disturbances of life history traits, enzyme activities or gene expression levels are observed. In particular, the link between changes of mRNA expression levels (transcriptomics) following exposure to chemical compounds and biochemical or physiological functions has been partially established in a limited number of soil invertebrates (Brulle et al., 2010). In *E. fetida* and *L. rubellus*, the consequence of experimental contaminant exposure on the level of expression of either selected genes, using real-time PCR, or of a large set of genes, using microarrays, has been extensively studied. In addition, most work has been carried out with the aim to describe dose–response changes (i.e. to highlight a relationship between gene expression levels and contamination levels) in worms exposed to stressful concentrations of various contaminants (Cd, fluoranthene and atrazine). Particular attention was paid to genes involved in detoxification, defense, anti-oxidant, DNA repair and metabolism of metal ions ( $\text{Fe}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Ca}^{2+}$ ) mechanisms. Usually, computer analyzes of the expression profiles confirm the involvement of genes controlling the mechanisms cited before. In this approach, the transcriptome is supposed to reflect the physiological state of the test species and is a relevant way to apprehend an initial stress in a dynamic manner (see Brulle et al., 2010).

In most organisms, including earthworms, physiological tolerance to metals depends, at least partially, on the induction of genes encoding metal binding proteins. Among these molecules, metallothioneins (MTs) are cysteine-rich proteins of low molecular weight (6000–8000 Da), characterized by the absence of aromatic amino acids, and known to be involved in detoxification processes of MTEs such as Cd and homeostasis of essential metals such as zinc (Palmiter, 1998; Klaassen et al., 1999). By binding essential and non-essential metal ions, MTs are involved in the homeostatic regulation and detoxification of metals (Klaassen et al., 1999; Brulle et al., 2006). For example, MTs provide detoxification function of some

non-essential metals such as Cd and protection during oxidative shock (Ghoshal et al., 1998; Baird et al., 2006). The physiological function of MTs has led several research teams to consider that they could be involved in the biological response to environmental stress following a contamination by a toxic chemical (Beattie et al., 2005). Accordingly, several research works were dedicated to the estimation of the impact of MTEs exposure based on the expression of MTs (or other proteins conferring resistance to metals) in various soil invertebrates. For instance, gene expression of a MT coding gene is induced by metals in earthworms species such as *E. fetida* (Demuyne et al., 2005, 2006) and *L. rubellus* (Stürzenbaum et al., 2004), but also in the nematode *C. elegans* (Liao and Freedman, 1999) and in the springtail *Orchesella cincta* (Timmermans et al., 2005). Induction of MTs during exposure to organic pollutants, although much less obvious, is also suggested (Kaegi, 1993). Consequently, in *L. rubellus* and in *E. fetida*, MT (in fact MT2 form) is considered as a good biomarker of exposure because the gene showed a time- and dose-dependent increase of both the number of MT2 transcripts and the protein concentration when worms are experimentally exposed to MTEs, especially Cd (Gruber et al., 2000; Brulle et al., 2006; Demuyne et al., 2007).

### 3. Interpreting the biological response of field-collected earthworms from sentinel species to MTE pollution

Experimental protocols of ecotoxicological studies interested in the characterization of the biological response of organisms to contaminant exposure are generally similar. Naïve specimens are exposed under control laboratory conditions, typically in microcosms (Fründ et al., 2010), to one or several levels of contaminant concentrations, using either artificially contaminated substrates or field sampled soils. It has to be noticed that, most of the time, exposed specimens belong to a few model species that may not naturally occur on polluted soils, but offer the advantage to be model species allowing the use of a large set of molecular tools. Moreover, those studies are mostly based on the analysis of stress responses over a short period of time at most equal to individual's lifetime. Whereas the accumulation of such a fundamental knowledge is crucial for the development of biomarkers, we have to consider that this may not be sufficient for two main reasons. First, because the biology of sentinel species used for biomonitoring may differ from the biology of model species used in the laboratory. Second, because field sampled organisms may belong to natural populations that have been exposed to contaminant over a long period of time, allowing an adaptive evolution of stress response.

- From model species to sentinel species.

The number of model species of macro-invertebrates that are commonly used in ecotoxicological experiments remains limited. Indeed, model species are usually limited to species that are easy to maintain and breed in laboratory conditions and for which molecular tools are available. On the contrary, criteria for selection of sentinel species can be quite different (O'Brien et al., 1993). Consequently, model and sentinel species may differ and biological and ecological differences among species may have significant consequences on their response to contaminant exposure. Considering the ecotoxicology of MTE exposure in oligochaete annelids, model species are mostly from the *Eisenia* genus. *E. fetida* and *E. andrei*, in particular, have been used in a majority of toxicological studies (Sanchez-Hernandez, 2006), although species from the *Lumbricus* genus are increasingly studied. In particular, *E. fetida* is the reference earthworm in the international toxicity tests (Nahmani et al., 2007b, 2007a). In contrast, candidate species for terrestrial ecosystem assessment in Central Europe belong to

several genera: *Eisenia*, but also *Lumbricus*, *Dendrodrius*, *Dendrobaena*, *Aporrectodea*, *Allolobophora*, *Proctodrilus*, *Octolasion* (Rombke et al., 2005; Lowe and Butt, 2007b, 2007c).

Experimental studies involving a direct or indirect comparison of the biological response of *Eisenia* species to other earthworm species (mostly from the *Lumbricus* genus) remain scarce but they suggest that putative physiological differences among species may only have a limited impact on their response to MTE exposure (Calisi et al., 2009, 2011). However, recent considerations suggest that the biological response of *E. fetida* may differ from other earthworm species because of ecological differences. Indeed, earthworms can be divided into three ecological groups (Table 1): epigeics, that live in the litter above the soil surface, anecics, that live in semi-permanent vertical deep burrows and come to the surface to feed, and endogeics, that rarely come to the soil surface, make horizontal non-permanent burrows and feed on the organic matter already in the soil (Bouché, 1992; Rombke et al., 2005). Thus, the ecological group to which a species belongs partly determines the exposure of the earthworms to contaminants. Because the horizontal distribution of MTEs in soil layers may be heterogenous, varying feeding behavior among soil dwelling earthworms occurring on a same polluted site may determine species-specific patterns of metal exposure and accumulation in tissues (Suthar et al., 2008). Accordingly, it can be shown that, at the community level, MTE pollution affects differently sympatric populations of endogeic and epigeic species (Nahmani et al., 2003). At the infra-individual level, uptake and bioaccumulation of MTEs may vary among earthworm ecological categories, with a general (but not systematic) tendency toward higher metal contents in tissues of epigeic and endogeic species than anecic species (Ernst et al., 2008; Suthar et al., 2008; Tischer, 2009). Finally, at the cellular level, the amount of Cd-induced DNA damage may also differ among ecological categories (Fourie et al., 2007).

Interestingly, the relative relevance of considering biological groups for the development of generic biomarkers may also be extended at the intra-specific level. For example, in *Allolobophora chlorotica*, two morphs have been described, depending on the presence of the biliverdin pigment (Satchell, 1967). *In situ* observations suggest that both morphs differ in ecological preferences related to soil moisture. The green form dominates in wet soils and pink form in dry soils. Laboratory experiments confirm that growth and maturation of the green form are significantly reduced in dry soil while growth of pink form is similar in dry or wet soil (Lowe and Butt, 2007a). Similarly, in *Lumbricus terrestris*, sequencing data of the cytochrome oxidase I (COI) mitochondrial gene revealed the existence of two cryptic species (James et al., 2010). Both species are supposed to live in sympatry, except in the north-west of France, where the exclusive presence of one cryptic species could be explained by ecological differentiation.

- From experimental populations to natural populations.

When analyzing the biological response to MTE exposure, shifting from experimentally exposed to field-sampled organisms may result in a significant difference in the history of contaminant exposure of organisms under study.

Basically, available space for earthworms in experimental conditions is generally highly reduced to mesocosm dimensions. In comparison, earthworms from natural populations that would be able to detect toxic compounds may migrate and escape from exposure (Capowiez et al., 2003, 2006). Thus, species-specific behavioral responses to contamination, especially the ability of earthworms to avoid soil pollutants should also be considered in the definition of biomarkers. Based on this behavior, the normalized

**Table 1**  
Ecological characteristics of relevant Central European earthworm species.

Ecological classification	Pigmentation	Feeding behavior	Adult size	Burrows	Mobility	Ecological role	Example
Epigeics	Heavy, usually both ventrally and dorsally.	Feed on organic matter in soil surface.	Small–medium	- Do not dig. - Live in the litter above the soil surface or in the upper reaches of the mineral soil.	Rapid movement in response to disturbance.	- Decomposing litter on the soil surface, little or no soil ingested.	- <i>Dendrobaena octaedra</i> - <i>Dendrobaena attemsi</i> - <i>Dendrobaena illyrica</i> - <i>Eisenia andrei</i> - <i>Eisenia fetida</i> - <i>Lumbricus rubellus</i> - <i>Lumbricus castaneus</i>
Anecic	Medium-heavy, usually only dorsally.	- Come to the surface to feed. - Feed on the organic matter on the surface and in the soil.	Large	- Make individual burrows that are vertical and deep.	Rapid withdrawal into burrow but more sluggish than epigeics.	- Their burrows affect the transfer of water, air and nutrients through the soil.	- <i>Aporrectodea nocturna</i> - <i>Lumbricus terrestris</i> - <i>Nicodrilus longus</i>
Endogeic	Unpigmented or lightly pigmented.	- Feed on the organic matter in the soil - Consume more soil than do others.	Medium	- Make horizontal non-permanent burrows in the soil.	Generally sluggish.	- Are responsible for the incorporation and distribution of organic matter in the soil.	- <i>Allolobophora chlorotica</i> - <i>Aporrectodea caliginosa</i> - <i>Aporrectodea rosea</i> - <i>Octolasion cyaneum</i> - <i>Proctodrilus oculata</i>

**Table 2**  
Referenced studies that previously suggested local adaptation in earthworm populations.

Species	Pollutant examined	Main results	References
<i>Dendrobaena octaedra</i>	Cd	Although Cd exposure generally affects life history and reproductive traits, the survival of F1 offspring of worms is significantly higher when the parents originate from polluted soil.	Rozen, 2006
<i>Dendrodrilus rubidus</i>	Cu	When exposed to soils treated with Cu, mortality of earthworm sampled from a Cu-polluted soil is shown to be lower than mortality of earthworms from a control Cu-free soil, suggesting that local adaptation leading to higher Cu-tolerance occurs in metalicolous populations of earthworms. Interestingly, the fertility of tolerant earthworm is significantly reduced, suggesting that Cu-tolerance has a cost.	Arnold et al., 2008
<i>Aporrectodea caliginosa</i> <i>Aporrectodea longa</i> <i>Aporrectodea rosea</i> <i>Dendrodrilus rubidus</i> <i>Lumbricus rubellus</i> <i>Lumbricus terrestris</i> <i>Octolasion cyaneum</i> <i>Octolasion tyrtaeum</i> <i>Lumbricus rubellus</i>	Hg, Cd and Pb	The study mainly focuses on interspecific comparisons from field-collected earthworms. However, results clearly suggest that intraspecific variation exists in both metal tissue concentrations and concentration factor. Investigating the genetic component of phenotypic variation would allow discussing the occurrence of genetic resources for metal tolerance evolution.	Ernst et al., 2008
	As	F1, F2, F3 offspring of worms collected in As-contaminated soils show better health after As exposure than offspring of worms from uncontaminated soils, suggesting the evolution of genetically based resistance to As-toxicity.	Langdon et al., 2009
<i>Dendrobaena octaedra</i>	Cu	F1-generation worms obtained in laboratory conditions are used to show higher growth rate of worms from contaminated sites in both control and copper-spiked soil conditions, suggesting adaptive population differentiation. However, no statistical difference among population in survival, maturation time, cocoon production and hatchability can be shown.	Fisker et al., 2011
<i>Eisenia fetida</i>	Pb, Cd	The total accumulated body burden of metals for worms homogeneously exposed to a contaminated field soil is highly variable among individuals. This suggests that genetic variation may occur in the molecular pathways of metal homeostasis.	Spurgeon et al., 2011
<i>Lumbricus rubellus</i>	As	Although not specifically addressed, genetic variation in response to As exposure is evidenced at several life stages. Population-level effects of As exposure are also discussed. Phenotypic variation for several life-history traits is observed among phylogenetically divergent clades in controlled conditions, suggesting that genetic resources allowing adaptation may exist. However, As sensitivity does not seem to differ among clades.	Anderson et al., 2013

avoidance test (ISO 17512-1 2008) has been developed and successfully used for different pesticides, mainly insecticides.

Moreover, experimental protocols of toxicological studies usually extend over short periods of time, typically inferior to an individual's lifetime. At this ontogenic timescale, mechanisms involved in stress response can only consist in physiological adjustments, a phenomenon known as acclimation or phenotypic plasticity (Morgan et al., 2007; Hendry et al., 2011). Thus, the over-expression of gene coding for proteins involved in detoxification processes illustrated above can be interpreted as an environmentally induced regulation of gene expression allowing single individuals to cope rapidly with a changing environment. On the contrary, organisms collected in contaminated sites may belong to natural populations that have been continuously exposed to contaminants for generations. In this case, long-term exposure, over many generations may have resulted in genetic changes conferring tolerance to toxic effects of metals in soil, leading to a genetic differentiation between exposed (or metalicolous) and non-exposed (or non-metallicolous) populations through a process called local adaptation (Kawecki and Ebert, 2004). This might bias estimations of the level of soil pollution from the phenotype of field-sampled organisms (Medina et al., 2007; Clements and Rohr, 2009; Bickham, 2011).

Local adaptation requires the action of selective pressures that are sufficiently strong and long lasting to (1) generate differential survivorship and reproductive success among individuals over several generations and therefore (2) when genetic polymorphisms exist, provoke changes in allelic or genotype frequencies in metalicolous natural populations compared to geographically close ones (Hendry et al., 2011). Considering the toxicity of MTE polluted soils and the persistence of MTEs (that cannot be degraded) in the environment, the evolution of local adaptation in metalicolous population of any species developing in close contact with a polluted soil is highly likely. A few studies carried out on species

from the soil macrofauna suggest it indeed occurs (Table 2). One of the best documented example concerns the soil-living collembola *Orchesella cincta*. In this species, it has been shown that the contamination of soils by MTEs modified the genetic composition of exposed populations, resulting in the evolution of more tolerant genotypes (Janssens et al., 2008; Costa et al., 2012).

In earthworms, definitive evidence of local adaptation of natural population to MTE exposure does not yet exist. However, several results (Table 2) suggest that (1) MTEs can cause varying fitness among individuals and thus act as selective pressures in natural populations and that (2) genetic variation, a fundamental condition for the evolution of local adaptation, exists for genes involved in homeostatic regulation and detoxification of metals.

### 3.1. Reduced fitness of earthworms under metal exposure

Negative consequence of MTEs exposure on fitness-related traits has been highlighted in several studies, suggesting that local adaptation can be assumed to occur in earthworm metalicolous natural populations. For example, using a specifically designed test system, Spurgeon et al. (2004) revealed exposure-dependent effects of copper and cadmium toxicity on survival, growth, development time of juveniles obtained from cocoons collected in natural populations of *L. rubellus*. Considering arsenic toxicity, the results were recently extended to adult life-history traits (Anderson et al., 2013). In another study focusing on *E. fetida*, Nahmani et al. (2007a) measured survival, body weight, cocoon production and hatching rate in earthworms exposed to metal-polluted and uncontaminated soils in control conditions. They revealed significant negative correlation among both growth and reproductive traits and metal concentration in soils, although soil characteristics other than metal concentration (organic carbon content, texture, pH) were shown to influence the level of soil toxicity.

### 3.2. Genetic resources for tolerance evolution in earthworms

The occurrence of genetic variation of metal-related genes is suggested by the varying concentrations of metals in tissues of conspecific earthworms either exposed to the same level of metal exposure in laboratory conditions (e.g. Smith et al., 2010) or collected at the same site (e.g. Ernst et al., 2008; Uba et al., 2009). However, in natural populations, varying concentrations of metals in tissues may also be due to the heterogeneity of metal exposure among sympatric earthworms. Thus, experimental exposure in controlled laboratory conditions is essential to evidence that among-individual variation in concentration of metals in tissues can be genetically determined (Lowe and Butt, 2007a, 2007b, 2007c). In *L. rubellus*, Spurgeon et al. (2011) specifically addressed the question of factors that could account for among individual variation for the total accumulated body burdens of Pb and Cd. By analyzing variation in individual tissue burdens of earthworms homogeneously exposed to metals, they concluded that observed differences might have a substantive biological component, suggesting that genetic variation exists for molecular mechanisms involved in uptake and/or elimination/biotransformation pathways. Interestingly, despite that, the genetic origin of phenotypic differences among metallicolous and non-metallicolous earthworms collected in the field can still be controversial. For example, Rozen (2006) showed that, in *Dendrobaena octaedra*, earthworms raised in similar laboratory conditions but collected in soils with various levels of metal pollution had different metal (Cd) body burdens, suggesting genetic differentiation. However, the same experiment on the offspring (F1 generation) of collected earthworms revealed no difference, suggesting that observed differences in cadmium accumulation ability among parental populations might not be heritable, and thus might reveal acclimatory adjustments rather than adaptive differences.

It is worth noting that genetically determined variations in the level of MTEs concentration in tissues may not necessarily mean that some genotypes or populations are more adapted to metaliferous sites than others. An experimental approach comparing the sensitivity to MTE exposure of populations from both polluted and unpolluted sites in controlled conditions would be appropriate to reveal a higher resistance of metaliferous population, i.e. a better fitness of those populations compared to population from uncontaminated environments (Janssens et al., 2008). In *Dendrodrius rubidus*, Arnold et al. (2008) compared the weight, health and mortality of earthworms from a mine spoil soil at an abandoned copper (Cu) mine and a Cu-free control site in several treatments varying for total Cu soil concentrations. In highly Cu-contaminated treatments, earthworms from mine sites were shown to be healthier and to suffer significantly less change in weight and mortality than control earthworms, suggesting higher resistance. Interestingly, the acquisition of resistance was assumed to be associated with an elevated physiological cost significantly reducing cocoon production and viability. In *L. rubellus*, Langdon et al. (2009) demonstrated that earthworms collected at an abandoned copper and arsenic (As) mine acquired genetically inherited resistance to As-toxicity. Following offspring of field-sampled earthworms exposed to 2000 mg As kg<sup>-1</sup> dry weight of soil over several generations, they showed that the former had lower mortality and higher cocoon viability than control earthworms. In *Dendrobaena octaedra*, Fisker et al. (2011) tested for the occurrence of adaptive differentiation for the level of copper among populations comparing three populations originating from copper contaminated sites to three originating from a non-contaminated site. For each population, F1-generations were produced, to ensure that among population differences were heritable, and placed in an uncontaminated control or copper-spiked soil. It

appeared from this experience that populations originating from polluted sites had a higher growth rate, an earlier acquisition of sexual maturity, a better reproduction rate and a lower mortality than populations originating from reference sites.

### 4. Biological responses of field collected earthworms at the transcriptome level

The evolution of local adaptation in natural earthworm populations occurring of metalliferous sites may imply that the biological response of field-collected earthworm could differ from the response of naïve specimens characterized in short-term laboratory experiments. At the transcriptome level, in particular, it may be expected than the inducible increase in the expression of genes involved in detoxification mechanisms observed in laboratory experiments (see above) cannot be confirmed in wild individuals from natural populations. Indeed, it seems that local adaptation of populations exposed to stressful conditions involves the evolution of constitutive over-expression of genes associated with stress response (Roelofs et al., 2008). Therefore, the level of expression of those genes may not be exclusively related to the level of exposure of individuals, as it is usually assumed in the biomarker concept focusing on transcriptomic tools.

In the specific context of MTE exposure, the constitutive over-expression of some target genes in adapted individuals of the springtail *O. cincta* comparing to sensitive ones has been demonstrated (Roelofs et al., 2008). In this species, increased resistance in natural populations has been shown to be associated with an elevated level of both constitutive and cadmium induced metallothionein expression (Sternborg and Roelofs, 2003), whereas individuals from sensitive populations show a typical stress-induced gene expression. It has been suggested that the evolution of over-expression could be related to modifications of the genetic composition of adapted populations through the increase in frequency of certain alleles for the promoter region of the MT gene. Interestingly, those alleles could be either shared with other populations or found exclusively in Cd-resistant populations (Costa et al., 2012). Similar results are not available in earthworms yet. However, it has been shown in a study testing the potential for the development of biomarkers in earthworms that the level of metallothionein expression observed in oligochaete annelids collected in a Cd-polluted site that has been polluted for more than 100 years was significantly increased (Péres et al., 2011).

### 5. Conclusion: integrating adaptation into the biomarker concept

Biomarkers have been mostly developed onto model species in short-term laboratory experiments, and transferred as such on sentinel species from natural populations. In this paper, it has been demonstrated that, in general, this could lead to misinterpretation owing to either (1) ecological and/or physiological differences among model and sentinel species or (2) the potential evolution of modified molecular mechanisms leading to local adaptation in natural populations that are exposed to contaminants over many generations. Considering available literature on earthworms, there is a real need for more comparative studies of the biology and physiology of model species (*Eisenia* sp.) and sentinel species to ensure that knowledge accumulated on *Eisenia* sp. can be transferred to other earthworm species. Moreover, it appears that conditions for local adaptation to occur are present in earthworms (namely, potential strong and durable selective pressures and within-population genetically determined phenotypic variability). In addition, some infra-individual evidence of adaptation obviously exists. Consequently, there is need for

evolutionary studies investigating the level and distribution of genetic diversity in natural populations of earthworms. In particular, the assumption of the evolution of constitutive over-expression of detoxification genes cannot be ruled out. Therefore, it seems necessary to integrate the potential effects of local adaptation to MTEs on biological responses of organisms in the development of biomarkers.

Identifying local adaptation and adaptive genes is a major task in evolutionary biology (Kawecki and Ebert, 2004; Barrett and Hoekstra, 2011). It is among the main objectives of an emerging discipline called “evolutionary ecotoxicology” (Coutellec and Barata, 2011), interested in determining the consequences of the exposure to anthropogenic contaminants on the evolution of natural populations. This mostly consists in studying the population genetics of natural populations differentially exposed to one or several contaminants and to test if among-population genetic changes observed at either the phenotypic or molecular levels may result from contaminant exposure. To infer local adaptation, it is also important to attest that observed changes affect fitness, *i.e.* the ability of organisms to survive and reproduce in their respective environment (Orr, 2009; Barrett and Hoekstra, 2011). For example, considering relationships between transcription levels of stress-response genes – such as MT – in local adaptation would require to confirm that (1) constitutive transcription levels vary among individuals, (2) variations are genetically determined and heritable (for example, by analyzing expression levels on offspring of field-samples individuals), (3) transcription levels affect tolerance to contaminants, and therefore fitness, (4) variations in transcription levels are not randomly distributed among exposed and unexposed populations.

Clearly, investigating local adaptation in earthworm would require the use of modern molecular technologies. For example, comparative transcriptomic studies would allow discussing whether molecular pathways involved in the adaptation of exposed natural population are similar to molecular pathways underlying the plastic response of naïve, *i.e.* model, organisms (namely *Eisenia* sp.) in laboratory conditions. Similarly, testing for adaptive genetic changes caused by contamination exposure in natural populations would require the use of molecular genotyping markers. In particular, one should ensure that individuals from exposed populations do not carry specific variants associated with constitutive over-expression of specific genes. In our opinion, this second point is critical since the availability of molecular markers remains low in earthworms (Dupont, 2009).

## References

- Anderson, C.J., Kille, P., Lawlor, A.J., Spurgeon, D.J., 2013. Life-history effects of arsenic toxicity in clades of the earthworm *Lumbricus rubellus*. *Environmental Pollution* 172, 200–207.
- Arnold, R.E., Hodson, M.E., Langdon, C.J., 2008. A Cu tolerant population of the earthworm *Dendrodrilus rubidus* (Savigny, 1862) at Coniston Copper Mines, Cumbria, UK. *Environmental Pollution* 152, 713–722.
- Baird, S.K., Kurz, T., Brunk, U.T., 2006. Metallothionein protects against oxidative stress-induced lysosomal destabilization. *Biochemical Journal* 394, 275–283.
- Barrett, R.D.H., Hoekstra, H.E., 2011. Molecular spandrels: tests of adaptation at the genetic level. *Nature Reviews Genetics* 12 (11), 767–780.
- Beattie, J.H., Owen, H.L.H., Wallace, S.M., Arthur, J.R., Kwun, I.S., Hawksworth, G.M., Wallace, H.M., 2005. Metallothionein overexpression and resistance to toxic stress. *Toxicology Letters* 157, 69–78.
- Beeby, A., 2001. What do sentinels stand for? *Environmental Pollution* 112, 285–298.
- Bickham, J.W., 2011. The four cornerstones of evolutionary toxicology. *Ecotoxicology* 20, 497–502.
- Bouché, M.B., 1992. Earthworm species and ecotoxicological studies. In: Greig-Smith, P.W., Becker, H., Edwards, P.J., Heimbach, F. (Eds.), *Ecotoxicology of Earthworms*. Intercept Press, Andover.
- 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., Cocquerelle, C., Mitta, G., Castric, V., Douay, F., Leprêtre, A., Vandenbulcke, F., 2008. Identification and expression profile of gene transcripts differentially expressed during metallic exposure in *Eisenia fetida* coelomocytes. *Developmental and Comparative Immunology* 32, 1441–1453.
- Brulle, F., Mitta, G., Cocquerelle, C., Vieau, D., Lemièrre, S., Leprêtre, A., Vandenbulcke, F., 2006. Cloning and real-time PCR testing of 14 potential biomarkers in *Eisenia fetida* following cadmium exposure. *Environmental Science & Technology* 40, 2844–2850.
- Brulle, F., Morgan, A.J., Cocquerelle, C., Vandenbulcke, F., 2010. Transcriptomic underpinning of toxicant-mediated physiological function alterations in three terrestrial invertebrate taxa: a review. *Environmental Pollution* 158, 2793–2808.
- Calisi, A., Lionetto, M.G., Schettino, T., 2009. Pollutant-induced alterations of granulocyte morphology in the earthworm *Eisenia fetida*. *Ecotoxicology and Environmental Safety* 72, 1369–1377.
- Calisi, A., Lionetto, M.G., Schettino, T., 2011. Biomarker response in the earthworm *Lumbricus terrestris* exposed to chemical pollutants. *Science of The Total Environment* 409, 4456–4464.
- Capowiez, Y., Bérard, A., 2006. Assessment of the effects of imidacloprid on the behavior of two earthworm species (*Aporrectodea nocturna* and *Alloobophora chlorotica*) using 2D terraria. *Ecotoxicology and Environmental Safety* 64, 198–206.
- Capowiez, Y., Rault, M., Mazzia, C., Belzunces, L., 2003. Earthworm behaviour as a source of biomarkers – a study case with imidacloprid. *Pedobiologia* 47, 542–547.
- Christensen, O., 1988. Lumbricid earthworms as bio-indicators relative to soil factors in different agro-ecosystems. *Advances in Management and Conservation of Soil Fauna*, 839–849.
- Clements, W.H., Rohr, J.R., 2009. Community responses to contaminants: using basic ecological principles to predict ecotoxicological effects. *Environmental Toxicology and Chemistry* 28, 1789–1800.
- Costa, D., Mariën, J., Janssens, T., van Gestel, C., Driessen, G., Sousa, J., van Straalen, N., Roelofs, D., 2012. Influence of adaptive evolution of cadmium tolerance on neutral and functional genetic variation in *Orchesella cincta*. *Ecotoxicology* 21, 2078–2087.
- Cotrufo, M.F., De Santo, A.V., Alfani, A., Bartoli, G., De Cristofaro, A., 1995. Effects of urban heavy metal pollution on organic matter decomposition in *Quercus ilex* L. Woods. *Environmental Pollution* 89, 81–87.
- Coutellec, M.A., Barata, C., 2011. An introduction to evolutionary processes in ecotoxicology. *Ecotoxicology* 20 (3), 493–496.
- Demuyck, S., Grumiaux, F., Descamps, M., Leprêtre, A., 2005. Differential expression of stress protein in response to heat and cadmium in two oligochaete species *Eisenia fetida* and *Eisenia andrei*. *Trends in Comparative Biochemistry & Physiology* 10, 105–110.
- Demuyck, S., Grumiaux, F., Mottier, V., Schikorski, D., Lemièrre, S., Leprêtre, A., 2006. Metallothionein response following cadmium exposure in the oligochaete *Eisenia fetida*. *Comparative Biochemistry and Physiology – C Toxicology and Pharmacology* 144, 34–46.
- Demuyck, S., Grumiaux, F., Mottier, V., Schikorski, D., Lemièrre, S., Leprêtre, A., 2007. Cd/Zn exposure interactions on metallothionein response in *Eisenia fetida* (Annelida, Oligochaeta). *Comparative Biochemistry and Physiology – C Toxicology and Pharmacology* 145, 658–668.
- Dickinson, N.M., Turner, A.P., Watmough, S.A., Lepp, N.W., 1992. Acclimation of trees to pollution stress: cellular metal tolerance traits. *Annals of Botany* 70, 569–572.
- Dupont, L., 2009. Perspectives on the application of molecular genetics to earthworm ecology. *Pedobiologia* 52, 191–205.
- Edwards, C.A., 1998. *Earthworm Ecology*. Soil and Water Conservation Society, St. Lucie Press, Iowa.
- Edwards, C.A., Bohlen, P.J., 1996. *Biology and Ecology of Earthworms*, third ed. Chapman & Hall, London.
- Ernst, G., Zimmermann, S., Christie, P., Frey, B., 2008. Mercury, cadmium and lead concentrations in different ecophysiological groups of earthworms in forest soils. *Environmental Pollution* 156, 1304–1313.
- Fisker, K., Sørensen, J., Damgaard, C., Pedersen, K., Holmstrup, M., 2011. Genetic adaptation of earthworms to copper pollution: is adaptation associated with fitness costs in *Dendrobaena octaedra*? *Ecotoxicology* 20, 563–573.
- Fourie, F., Reinecke, S.A., Reinecke, A.J., 2007. The determination of earthworm species sensitivity differences to cadmium genotoxicity using the comet assay. *Ecotoxicology and Environmental Safety* 67, 361–368.
- Fründ, H.C., Butt, K., Capowiez, Y., Eisenhauer, N., Emmerling, C., Ernst, G., Potthoff, M., Schadler, M., Schrader, S., 2010. Using earthworms as model organisms in the laboratory: recommendations for experimental implementations. *Pedobiologia* 53, 119–125.
- Ghalambor, C.K., McKay, J.K., Carroll, S.P., Reznick, D.N., 2007. Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. *Functional Ecology* 21, 394–407.
- Ghoshal, K., Wang, Y., Sheridan, J.F., Jacob, S.T., 1998. Metallothionein induction in response to restraint stress. Transcriptional control, adaptation to stress, and role of glucocorticoid. *Journal of Biological Chemistry* 273, 27904–27910.
- Gruber, C., Stürzenbaum, 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*. *European Journal of Biochemistry* 267, 573–582.

- Hendry, A.P., Kinnison, M.T., Heino, M., Day, T., Smith, T.B., Fitt, G., Bergstrom, C.T., Oakeshott, J., Jorgensen, P.S., Zalucki, M.P., Gilchrist, G., Southerton, S., Sih, A., Strauss, S., Denison, R.F., Carroll, S.P., 2011. Evolutionary principles and their practical application. *Evolutionary Applications* 4 (2), 159–183.
- Hopkin, S.P., 1989. *Ecophysiology of Metals in Terrestrial Invertebrates*. Elsevier Applied Science, London.
- ISO (International Organisation for Standardization) 17512-1, 2008. *Soil Quality – Avoidance Test for Determining the Quality of Soils and Effects of Chemicals on Behaviour – Part 1: Test with Earthworms (*Eisenia fetida* and *Eisenia andrei*)*.
- James, S.W., Porco, D., Decaens, T., Richard, B., Rougerie, R., Erseus, C., 2010. DNA barcoding reveals cryptic diversity in *Lumbricus terrestris* L, 1758 (Clitellata): resurrection of *L. herculeus* (Savigny, 1826). *PLoS ONE* 5, e15629.
- Janssens, T.K.S., Lopez, R.D.R., Marien, J., Timmermans, M., Montagne-Wajer, K., Van Straalen, N.M., Roelofs, D., 2008. Comparative population analysis of metallothionein promoter alleles suggests stress-induced microevolution in the field. *Environmental Science & Technology* 42, 3873–3878.
- Kaegi, J.H.R., 1993. Evolution, structure and chemical activity of class I metallothioneins: an overview. *Metallothionein III*, 29–55.
- Kautenburger, R., 2006. Impact of different agricultural practices on the genetic structure of *Lumbricus terrestris*, *Arion lusitanicus* and *Microtus arvalis*. *Animal Biodiversity and Conservation* 29, 19–32.
- Kawecki, T.J., Ebert, D., 2004. Conceptual issues in local adaptation. *Ecology Letters* 7 (12), 1225–1241.
- Klaassen, C.D., Liu, J., Choudhuri, S., 1999. Metallothionein: an intracellular protein to protect against cadmium toxicity. *Annual Review of Pharmacology and Toxicology* 39, 267–294.
- Kozlov, M.V., Zvereva, E.L., 2011. A second life for old data: global patterns in pollution ecology revealed from published observational studies. *Environmental Pollution* 159, 1067–1075.
- Langdon, C.J., Morgan, A.J., Charnock, J.M., Semple, K.T., Lowe, C.N., 2009. As-resistance in laboratory-reared F1, F2 and F3 generation offspring of the earthworm *Lumbricus rubellus* inhabiting an As-contaminated mine soil. *Environmental Pollution* 157, 3114–3119.
- Lavelle, P., Spain, A.V., 2001. *Soil Ecology*. Kluwer Academic Publishers, Dordrecht, Spain.
- Liao, V.H.C., Freedman, J.H., 1999. Cadmium-regulated genes from the nematode *Caenorhabditis elegans*: identification and cloning of new cadmium-responsive genes by differential display. *Journal of Biological Chemistry* 273, 31962–31970.
- Lowe, C.N., Butt, K.R., 2007a. Life-cycle traits of the dimorphic earthworm species *Allolobophora chlorotica* (Savigny, 1826) under controlled laboratory conditions. *Biology and Fertility of Soils* 43, 495–499.
- Lowe, C.N., Butt, K.R., 2007b. Culture of commercially obtained *Lumbricus terrestris* L.: implications for sub-lethal ecotoxicological testing. *Soil Biology and Biochemistry* 39 (7), 1674–1679.
- Lowe, C.N., Butt, K.R., 2007c. Earthworm culture, maintenance and species selection in chronic ecotoxicological studies: a critical review. *European Journal of Soil Biology* 43, S281–S288.
- Lukkari, T., Taavitsainen, M., Väisänen, A., Haimi, J., 2004. Effects of heavy metals on earthworms along contamination gradients in organic rich soils. *Ecotoxicology and Environmental Safety* 59, 340–348.
- Manios, T., Stentiford, E.L., Millner, P.A., 2003. The effect of heavy metals accumulation on the chlorophyll concentration of *Typha latifolia* plants, growing in a substrate containing sewage sludge compost and watered with metaliferous water. *Ecological Engineering* 20, 65–74.
- Marhan, S., Scheu, S., 2005. The influence of mineral and organic fertilisers on the growth of the endogeic earthworm *Octolasion tyrtaeum* (Savigny). *Pedobiologia* 49, 239–249.
- Medina, M.H., Correa, J.A., Barata, C., 2007. Micro-evolution due to pollution: possible consequences for ecosystem responses to toxic stress. *Chemosphere* 67, 2105–2114.
- Moffett, B.F., Nicholson, F.A., Uwakwe, N.C., Chambers, B.J., Harris, J.A., Hill, T.C.J., 2003. Zinc contamination decreases the bacterial diversity of agricultural soil. *FEMS Microbiology Ecology* 43, 13–19.
- Morgan, A.J., Kille, P., Sturzenbaum, S.R., 2007. Microevolution and ecotoxicology of metals in invertebrates. *Environmental Science & Technology* 41, 1085–1096.
- Müller, A.K., Westergaard, K., Christensen, S., Sørensen, S.J., 2001. The effect of long-term mercury pollution on the soil microbial community. *FEMS Microbiology Ecology* 36, 11–19.
- Nahmani, J., Hodson, M.E., Black, S., 2007a. Effects of metals on life cycle parameters of the earthworm *Eisenia fetida* exposed to field-contaminated, metal-polluted soils. *Environmental Pollution* 149, 44–58.
- Nahmani, J., Hodson, M.E., Black, S., 2007b. A review of studies performed to assess metal uptake by earthworms. *Environmental Pollution* 145, 402–424.
- Nahmani, J., Lavelle, P., Lapiéd, E., van Oort, F., 2003. Effects of heavy metal soil pollution on earthworm communities in the north of France: the 7th international symposium on earthworm ecology. Cardiff, Wales. 2002. *Pedobiologia* 47, 663–669.
- Nawrot, T., Plusquin, M., Hogervorst, J., Roels, H.A., Celis, H., Thijs, L., Vangronsveld, J., Van Hecke, E., Staessen, J.A., 2006. Environmental exposure to cadmium and risk of cancer: a prospective population-based study. *Lancet Oncology* 7, 119–126.
- Obrien, D.J., Kaneene, J.B., Poppenga, R.H., 1993. The use of mammals as sentinels for human exposure to toxic contaminants in the environment. *Environmental Health Perspectives* 99, 351–368.
- Orr, H.A., 2009. Fitness and its role in evolutionary genetics. *Nature Reviews Genetics* 10 (8), 531–539.
- Palmiter, R.D., 1998. The elusive function of metallothioneins. *Proceedings of the National Academy of Sciences of the United States of America* 95, 8428–8430.
- Peralta-Video, J.R., Lopez, M.L., Narayan, M., Saupe, G., Gardea-Torresdey, J., 2009. The biochemistry of environmental heavy metal uptake by plants: implications for the food chain. *International Journal of Biochemistry and Cell Biology* 41, 1665–1677.
- Péres, G., Vandembulcke, F., Guernion, M., Hedde, M.L., Beguiristain, T., Douay, F., Houot, S., Piron, D., Richard, A., Bispo, A., Grand, C.C., Galsomies, L., Cluzeau, D., 2011. Earthworm indicators as tools for soil monitoring, characterization and risk assessment. An example from the national bioindicator programme (France). *Pedobiologia* 54 (Suppl.), S77–S87.
- Pearce, T.G., Langdon, C.J., Meharg, A.A., Semple, K.T., 2002. Yellow earthworms: distinctive pigmentation associated with arsenic- and copper-tolerance in *Lumbricus rubellus*. *Soil Biology and Biochemistry* 34, 1833–1838.
- Pigliucci, M., 2005. Evolution of phenotypic plasticity: where are we going now? *Trends in Ecology and Evolution* 20, 481–486.
- Pizl, V., Jøsen, G., 1995. Earthworm communities along a gradient of urbanization. *Environmental Pollution* 90, 7–14.
- Ricketts, H.J., Morgan, A.J., Spurgeon, D.J., Kille, P., 2004. Measurement of annetocin gene expression: a new reproductive biomarker in earthworm ecotoxicology. *Ecotoxicology and Environmental Safety* 57, 4–10.
- 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. *Functional Ecology* 22, 8–18.
- Rombke, J., Jansch, S., Didden, W., 2005. The use of earthworms in ecological soil classification and assessment concepts. *Ecotoxicology and Environmental Safety* 62, 249–265.
- 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 Biology and Biochemistry* 38, 489–503.
- Sanchez-Hernandez, J.C., 2006. Earthworm biomarkers in ecological risk assessment. *Reviews of Environmental Contamination and Toxicology* 188, 85–126.
- Satchell, J.E., 1967. Lumbricidae. In: Burges, A., Raw, F. (Eds.), *Soil Biology*. Academic Press, London New York, pp. 259–322.
- Seeber, J., Seeber, G.U.H., Kössler, W., Langel, R., Scheu, S., Meyer, E., 2005. Abundance and trophic structure of macro-decomposers on alpine pastureland (Central Alps, Tyrol): effects of abandonment of pasturing. *Pedobiologia* 49, 221–228.
- Smith, B., Egeler, P., Gilberg, D., Hendershot, W., Stephenson, G., 2010. Uptake and elimination of cadmium and zinc by *Eisenia andrei* during exposure to low concentrations in artificial soil. *Archives of Environmental Contamination and Toxicology* 59, 264–273.
- Spurgeon, D.J., Lister, L., Kille, P., Pereira, M.G., Wright, J., Svendsen, C., 2011. Toxicokinetic studies reveal variability in earthworm pollutant handling. *Pedobiologia* 54 (Suppl.), S217–S222.
- Spurgeon, D.J., Svendsen, C., Kille, P., Morgan, A.J., Weeks, J.M., 2004. Responses of earthworms (*Lumbricus rubellus*) to copper and cadmium as determined by measurement of juvenile traits in a specifically designed test system. *Ecotoxicology and Environmental Safety* 57, 54–64.
- 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.
- Sterenborg, I., Roelofs, D., 2003. Field-selected cadmium tolerance in the springtail *Orchesella cincta* is correlated with increased metallothionein mRNA expression. *Insect Biochemistry and Molecular Biology* 33, 741–747.
- Stork, N.E., Eggleton, P., 1992. Invertebrates as determinants and indicators of soil quality. *American Journal of Alternative Agriculture* 7, 38–45.
- Stürzenbaum, S.R., Georgiev, O., Morgan, A.J., Kille, P., 2004. Cadmium detoxification in earthworms: from genes to cells. *Environmental Science & Technology* 38, 6283–6289.
- Suthar, S., Singh, S., Dhawan, S., 2008. Earthworms as bioindicator of metals (Zn, Fe, Mn, Cu, Pb and Cd) in soils: Is metal bioaccumulation affected by their ecological category? *Ecological Engineering* 32, 99–107.
- 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.
- Tischer, S., 2009. Earthworms (Lumbricidae) as bioindicators: the relationship between in-soil and in-tissue heavy metal content. *Polish Journal of Ecology* 57, 513–523.
- Uba, S., Uzairu, A., Okunola, O.J., 2009. Content of heavy metals in *Lumbricus terrestris* and associated soils in dump sites. *International Journal of Environmental Research* 3, 353–358.
- Van Der Schalie, W.H., Gardner Jr., H.S., Bantle, J.A., De Rosa, C.T., Finch, R.A., Reif, J.S., Reuter, R.H., Backer, L.C., Burger, J., Folmar, L.C., Stokes, W.S., 1999. Animals as sentinels of human health hazards of environmental chemicals. *Environmental Health Perspectives* 107, 309–315.
- van Gestel, C.A.M., van Brummelen, T.C., 1996. Incorporation of the biomarker concept in ecotoxicology calls for a redefinition of terms. *Ecotoxicology* 5, 217–225.
- Van Kleunen, M., Fischer, M., 2005. Constraints on the evolution of adaptive phenotypic plasticity in plants. *New Phytologist* 166, 49–60.
- Van Straalen, N.M., 1998. Evaluation of bioindicator systems derived from soil arthropod communities. *Applied Soil Ecology* 9, 429–437.
- Weeks, J.M., 1995. The value of biomarkers for ecological risk assessment: academic toys or legislative tools? *Applied Soil Ecology* 2, 215–216.