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Earthworm Biomarkers in Ecological Risk Assessment

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Contents

I. Introduction

Earthworms are important components of the soil system, mainly because of their favorable effects on soil structure and function (Paoletti 1999; Jongmans et al. 2003). Their burrowing and feeding activities contribute notably to increased water infiltration, soil aeration, and the stabilization of soil aggregates. In addition, earthworms help to increase soil fertility by formation of an organic matter layer in topsoil. These features, among others, have led to the popularity of earthworms as excellent bioindicators of soil pollution (Cortet et al. 1999; Lanno et al. 2004). These organisms ingest large amounts of soil, or specific fractions of soil (i.e., organic matter), thereby being continuously exposed to contaminants through their alimentary surfaces (Morgan et al. 2004). Moreover, several studies have shown that earthworm skin is a significant route of contaminant uptake as well (Saxe et al. 2001; Jager et al. 2003; Vijver et al. 2005).

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Several earthworm species (e.g., *Eisenia fetida* and *E. andrei*) have occupied an important place in toxicity testing (OECD 1984). The primary goals of these tests have been (i) the assessment of potential toxicity of new chemicals to be introduced into the environment, and (ii) the risk assessment for toxic effects from historically contaminated soils. Earthworms have also been used as bioindicators in the field monitoring of soil pollution. Changes in abundance, biomass, or species richness of natural populations have been common ecological endpoints to identify point-sources of pollution (Spurgeon and Hopkin 1999; Nahmani and Lavelle 2002; Dunger and Voigtländer 2005; Vandecasteele et al. 2004). Their tolerance to highly metal-contaminated soils and capacity to accumulate elevated concentrations of heavy metals in their tissues have led to the use of earthworms as sentinel species (Lukkari et al. 2004a; Carpené et al. 2006).

Earthworm biomarkers have scarcely been investigated, particularly under field conditions. Some of them (e.g., lysosomal fragility) have received particular attention in recent years. Generally, the term "biomarker" is easily exchangeable by "bioindicator" in the ecotoxicological literature and can lead to the reader's confusion. In this review, a biomarker or biological marker refers to any biological response (from molecular to behavioral changes) to one or more contaminants (Peakall 1992; Lagadic et al. 2000; Walker et al. 2001; Handy et al. 2003; Vasseur and Cossu-Leguille 2003). The term bioindicator, however, defines an organism that gives information on the environmental conditions of its habitat by its presence or absence (van Gestel and van Brummelen 1996). Most authors agree that biomarkers are sensitive indicators of contaminant exposure, whose main goal is to serve as early warning signs of predictive adverse effects at higher biological organization levels (population or community). To date, however, biomarkers provide an indication of exposure only. Thus, the determination of multiple biomarkers across different levels of biological organization is recommended to provide a better assessment of ecological consequences of contamination (Spurgeon et al. 2005a). Recently, biomarkers have gained ecotoxicological meaning when they have been integrated in an ecological weight-of-evidence (WOE) framework (Neuparth et al. 2005).

Two international meetings held in Denmark (3rd International Workshop on Earthworm Ecotoxicology; special issue of *Ecotoxicology and Environmental Safety*, vol. 57, 2004) and UK (7th International Symposium on Earthworm Ecology; special issue of *Pedobiology*, vol. 47, 2003) have examined the current knowledge of earthworm ecotoxicology. Previously, two exhaustive reviews summarized the available information on the most common earthworm biomarkers (Kammenga et al. 2000; Scott-Fordsmand and Weeks 2000). Some remarkable conclusions can be drawn from these reviews. Although a broad group of molecular biomarkers such as cholinesterases (ChEs), cytochrome P450-dependet monooxygenases, DNA breakage, or enzymes of oxidative stress have been traditionally measured in earthworms, they have been mainly studied in response to

heavy metal exposure (Cd, Cu, Pb, Zn). Therefore, there is a need for developing biomarkers of exposure/effects to organic contaminants of current concern (e.g., polycyclic aromatic hydrocarbons, pesticides, polybrominated flame retardants) or even other metals such as mercury. Furthermore, some of the features that define an ideal biomarker have yet not been investigated in the earthworm. For example, impact of confounding factors (environmental and biological) on biomarker responses and their normal variations need to be investigated (Scott-Fordsmand and Weeks 2000). A set of recommendations drawn from the 3rd International Workshop on Earthworm Ecotoxicology (van Gestel and Weeks 2004) can be summarized in the following points:

It is necessary to investigate the toxicodynamic (i.e., mechanism of toxicity at the target site) of chemicals to develop new, sensitive, and reliable biomarkers.

Biomarkers should be examined under field conditions to validate them as early warning indicators of negative ecological consequences.

Biomarker responses must be linked to adverse effects on life cycle traits (cocoon production rate or changes in body weight) under laboratory bioassays.

It is necessary to assess the impact of environmental factors (e.g., temperature, pH, osmotic stress, organic matter content, or photoperiod) and biological variables (e.g., reproductive cycle, nutritional status) on the biomarkers.

Most of the research on earthworm biomarkers involves the effects of certain heavy metals only (e.g., Cu, Cd, Zn or Pb), and investigations on biomarker responses to organic pollutant exposure are rather scarce.

The purpose of this review is to examine the current knowledge on earthworm biomarkers, as well as the application of biomarkers in ecological risk assessment (ERA) of contaminated soils. A critical discussion, organized in three sections, undertakes (1) the potential use of earthworm biomarkers as sublethal endpoints in standardized toxicity tests, (2) the main drawbacks in the assessment procedures of contaminated soils, and (3) the use of earthworm biomarkers for assessing the effectiveness of two procedures currently applied for recovering/protecting the environment: the soil bioremediation and the agrienvironment schemes, implemented in many countries of the European Union. Finally, future lines of research are suggested to increase the understanding of earthworm biomarkers.

II. Earthworm Biomarkers

A. Ecotoxicological Tests

Toxicity tests constitute an essential element of the ERA scheme (exposure and effect assessment). They are used to predict acute and/or chronic effects of new chemicals before release into the environment or to assess the

ecological impact of a new aqueous or atmospheric emission sources (predictive ERA). Similarly, ecotoxicity assays are also used in a retrospective approach of ERA to assess the historical contamination with possible ongoing ecological consequences. In general terms, toxicity testing has been the main instrument for legal requirements and environmental management decisions, which has led to the development of multiple standardized protocols depending mainly on the ambient media or test organism. An extensive description of toxicity tests used for aquatic environment assessments is compiled in the textbook *Fundamentals of Aquatic Toxicology* (Rand 2003). A guideline for conducting soil toxicity tests has been reported by the Organisation for Economic Co-operation and Development (OECD 1984, 2004) or by the International Standard Organization (ISO 1993, 1998, 2004). A description of the most common soil toxicity tests is available in van Straalen and van Gestel (1998) or Jänsch et al. (2005).

The typical endpoints in any standardized acute or chronic toxicity test are survival, reproduction rate, growth, or immobilization (e.g., daphnids). When field-contaminated soils or sediments are used to assess their toxicity (retrospective ERA), uncertainties in the test results can be associated to factors other than the contaminant burden present in the environmental media. The application of the appropriate biomarkers could provide further information about the active bioavailable fraction of the contaminant (Lanno et al. 2004). Moreover, biomarkers can give clear evidence of a cause–effect relationship between the contaminant in the environmental media and the occurrence of adverse effects at the individual level. Sediment toxicology, for instance, has been initiated to integrate certain molecular biomarkers in acute toxicity tests to assess sublethal toxic effects at multiple levels of biological organization (Neuparth et al. 2005). This current tendency is also becoming a common practice in soil toxicity tests using earthworms. This review does not attempt to give an exhaustive compilation of the earthworm toxicity assays but describes only those studies in which biomarkers have been integrated in the suite of toxicological endpoints.

The measurement of lysosomal membrane stability through the neutral red retention (NRR) assay, which combines analytical simplicity and ecological realism (complexity), has become one of the most popular earthworm biomarkers. The NRR assay in earthworms was first described by Weeks and Svendsen (1996); a review of their qualities was published by Svendsen et al. (2004). The NRR assay is determined in coelomocytes collected from the coelomic fluid. The quantification of this biomarker response implies the measurement of the time required to achieve 50% stained cells of the total cells counted periodically under a light microscope during a fixed time period. Lysosomal membrane stability can decrease in response to stress, and this is manifested in the NRR assay as a gradual leak of the neutral red from the lysosomes into the surrounding cytoplasm.

Damage in the lysosomal membrane caused by contaminant exposure is associated, therefore, with a decrease in the NRR time with respect to that in intact lysosomes.

Some studies have demonstrated that this biomarker is a useful predictor of adverse effects on life cycle traits (e.g., survival, growth, or reproduction). For example, Svendsen and Weeks (1997a) found that NRR times in *E. andrei* exposed to Cu were significantly reduced when metal concentration in soil was 20 mg kg^{-1}, whereas survival or changes in body weight were significantly affected at Cu concentrations as high as 320 mg kg^{-1} (Table 1). Similarly, Booth and O'Halloran (2001) reported that the NRR assay in adult earthworms (*Aporrectodea caliginosa*) exposed for 28 d to sublethal concentrations of the organophosphate insecticides diazinon and chlorpyrifos was a more-sensitive indicator than growth rate or cocoon production. Exposure to Pb also caused a significant and concentration-dependent reduction in the NRR time of *E. fetida* after 4 wk of metal exposure (Booth et al. 2003). A negative linear correlation was found between the logarithmic-transformed Pb concentrations in the earthworm body and the NRR times. This earthworm species also showed a substantial decrease of the NRR time up to 4 min (NRR times were ~50 min in control group) after exposure to Cu concentrations higher than 300 mg kg^{-1} (Scott-Fordsmand et al. 2000). In this study, the reduction in NRR time corresponded to an earthworm body Cu concentration of about 50 mg kg^{-1}. This is a clear example of why internal metal concentration is a more reliable endpoint than traditional external metal concentration, especially when parameters such as EC_{50} are estimated (Escher and Hermens 2004). Nevertheless, the internal metal concentration does not reflect the bioactive fraction (internal effect concentration). The toxicant concentration or dose at target site (bioactive fraction) can be estimated from models based on simple partitioning or more complex kinetics (Escher and Hermens 2004). Biomarkers such as the NRR assay might be a useful tool for estimating the internal effect concentration because they reflect the bioactive contaminant fraction.

The historical use of earthworms as biomonitors of metal soil pollution has contributed notably to the characterization of metallothioneins (MTs) in these organisms. These low molecular weight and cysteine-rich proteins have been isolated and fully characterized in *Lumbricus rubellus* (Stürzenbaum et al. 1998) and *E. fetida* (Gruber et al. 2000). In the case of *L. rubellus*, two MT isoforms (i.e., wMT-1 and wMT-2) have been isolated and seem to have different physiological functions and responses to metal exposure (Stürzenbaum et al. 1998; Morgan et al. 2004). wMT-2 has been the MT isoform more studied in relation to metal exposure because of its role in heavy metal sequestration. It shows a marked induction in *L. rubellus* exposed to increasing Cd or Cu concentrations in soil (Burgos et al. 2005; Spurgeon et al. 2005b).

Table 1. Biomarker responses in earthworms experimentally exposed to organic pollutants or heavy metals.

Species	Test substance: chemical form	Concentration and time of exposure	Test conditions	Biomarkers	Main remarks	Reference[a]
Eisenia fetida andrei (clitellate adults)	Carbaryl	12, 25, and 50 mg kg^{-1} 2, 7, and 14 d	Artificial soil (OECD) Soil moisture = 35% Temp. = 20° ± 1°C pH = 6.5 ± 0.5 Continuous light	AChE MROD NADH Red NADPH Red Catalase GR GST LP and LPI Total GSH % GSSG	Significant depression of enzymes involved in biotransformation of xenobiotics (MROD, NADH Red, and NADPH Red), but lack of a consistent concentration–response relationship. Significant inhibition of AChE activity in all treatments and time of exposure.	(1)
E. fetida andrei (clitellate adults)	Pb: lead acetate	30, 60, 120, and 250 mg kg^{-1} 2, 7, 14, and 28 d	Artificial soil (OECD) Soil moisture = 35% Temp. = 20° ± 1°C pH = 5.8–6.20 Continuous light	AChE MROD NADH Red NADPH Red Catalase GR GST LP and LPI Total GSH % GSSG	Induction of lipid peroxidation but related to neither Pb concentration nor time of exposure. Depression of GST at all treatments after 2 d of exposure. Significant variations of enzymes involved in biotransformation process but no clear relationships with Pb concentrations were observed. Reactive oxygen species (ROS) probably generated after Pb exposure.	(2)
E. fetida andrei (clitellate adults)	Benzo(*a*)pyrene	0.05, 1, 100, and 1,000 mg kg^{-1} 1, 2, 7, and 14 d	Artificial soil (OECD) Soil moisture = 35% Temp. = 20° ± 1°C pH = 6.5 ± 0.5 Continuous light	AChE MROD NADH Red NADPH Red Catalase GR GST LP and LPI Total GSH % GSSG	Induction of MROD activity at low B(a)P concentrations, but inhibition at high (>100 mg kg^{-1}) concentrations. Induction of lipid peroxidation and increase of several enzyme activities (AChE, catalase), but no evident relationship with B(a)P concentrations or time of exposure. ROS probably involved in B(a)P exposure.	(3)

Table 1. *Continued*

Species	Test substance: chemical form	Concentration and time of exposure	Test conditions	Biomarkers	Main remarks	Reference[a]
E. foetida	Chlorpyrifos	2.96 ± 0.39, and 2.33 ± 0.39 mg ml^{-1} 12, 24, 36, and 48 hr	Filter paper contact test (OECD, method 207)	AChE	Significant inhibition (>60%) of AChE activity at the two pesticide concentrations. Morphological abnormalities (necrosis and damage of the structure of muscles, bloody lesions, rectal areas detached).	(4)
Drawida willsi (juveniles)	Butachlor, Malathion and Carbofuran	1.1. and 2.2 mg kg^{-1} (butachlor and carbofuran) 2.2 and 4.4 mg kg^{-1} (malathion) Perodically from 1 to 105 d	Natural soil Soil moisture = 20% Temp. = 25° ± 2°C pH = 6.8	AChE	No variations of AChE activity in earthworms exposed to butachlor. Maximum AChE inhibition (41% and 46%) after 9 d of malathion exposure, and after 12 d (54% and 62.9%) of carbofuran exposure. AChE recovered its normal activity after 45 d of malathion exposure, while it needed 75 d to full activity recovery under carbofuran exposure.	(5)
Lumbricus rubellus (adults)	Pyrene	10, 40, 160, 640, and 2,560 mg kg^{-1} 42 d	Artificial sterilized loam soil Soil moisture = 80% Temp. = 15° ± 1.5°C 16 : 8 hr, L : D cycle	EROD Catalase	No EROD activity was detected. Catalase activity was lower in earthworms exposed to 160 and 640 mg kg^{-1} respect to control group.	(6)
Aporrectodea tuberculata (clitellate adults)	Cu : $CuCl_2 \cdot 2H_2O$ Zn: $ZnCl_2$	Cu/Zn = 100/175, 200/350, and 400/700 mg kg^{-1} 2, 7, and 14 d	Natural soil (collected from Jyväskylä, a nonpolluted area in Finland) Soil moisture = 26% ± 4% pH = 6.7 ± 0.1 Temp. = 15°C Darkness	CYP1A MT GST	Concentration-dependent induction of MT in earthworms exposed to Cu and Zn after 2 and 7 d of exposure. Earthworm populations naturally exposed to metal-contaminated soils showed a higher MT induction than not naturally metal-exposed population after 2 and 7 d of exposure to Cu/Zn-spiked soils. Induction of CYP1A activity after Cu and Zn exposure.	(7)

Table 1. *Continued*

Species	Test substance: chemical form	Concentration and time of exposure	Test conditions	Biomarkers	Main remarks	Reference[a]
E. fetida (clitellate adults)	Zn and Pb	Reference soil: Zn = 1.75 ± 0.25 μg g^{-1} Pb = 4.30 ± 0.99 μg g^{-1} Metalliferous soil: Zn > 1,500 μg g^{-1} Pb > 500 μg g^{-1} 20 d	Reference soil: commercial Kettering loam pH = 6.1 Organic matter = 7.0% Metalliferous soil: collected at an abandoned Zn/Pb mine pH = 6.4 Organic matter = 35% Temp. = 15° ± 1°C Soil moisture = 75% 12 : 12 hr, L : D cycle	Annetocin	A 20-fold reduction in the annetocin expression was found in earthworms exposed to the metalliferous soil respect to those exposed to the reference soil.	(8)
E. andrei (clitellate adults)	Cu: $CuCl_2 \cdot 2H_2O$	Cu = 20, 40, 80, 160, and 320 mg kg^{-1} 28 d	Field soils Soil moisture = 50% pH = 5.6 Organic matter <1% Temp. = 15°C Constant light	NRR assay	Significant dose-related decrease in the NRR time with soil and body Cu concentrations. Suggested toxic threshold for the Cu of 40–80 mg kg^{-1}. NRR assay becomes a more sensitive indicator than mortality or individual growth.	(9)
E. fetida (clitellate adults)	Cu: $CuCl_2 \cdot 2H_2O$	Cu = 50, 100, 300, 700, and 1400 mg kg^{-1} 21 d	Natural soil. pH = 6.5–7.0 Temp. = 20° ± 1°C 12 : 12 h, L : D cycle	NRR assay	Earthworms exposed to soils spiked with 300 mg kg^{-1} showed a NRR time <4 min, indicating a significant toxic effect. A range of NRR times between 4 and 6 min corresponded to an internal Cu concentration >50 mg kg^{-1}.	(10)

Table 1. *Continued*

Species	Test substance: chemical form	Concentration and time of exposure	Test conditions	Biomarkers	Main remarks	Reference[a]
E. fetida (clitellate adults)	Pb: $Pb(NO_3)_2$	Pb = 20, 40, 80, 160, and 320 mg kg^{-1} 28 d	Sieved cattle manure Soil moisture = 75% pH = 7.0 Temp. = 25°C	NRR assay	Decrease of NRR time with increasing of Pb body burden.	(11)
Aporrectodea caliginosa (adults and juveniles)	Diazinon: Basudin 600EW, 60% a.i. Chlorpyrifos: Lorsban 40EC, 40% a.i.	Diazinon = 12 and 60 mg kg^{-1} Chlorpyrifos = 4 and 28 mg kg^{-1} 28 d	Natural soil. Soil moisture = 25% pH = 6.5–7.0 Temp. = 20°C Constant light	NRR assay ChE GST	ChE activity was drastically inhibited in juvenile earthworms exposed to 12 (75% inhibition) and 60 (90%) mg kg^{-1} of diazinon. Chlorpyrifos caused a ChE depression of 35% and 70% respect to controls in earthworms exposed to 4 and 28 mg kg^{-1}, respectively. GST activity was inhibited by both diazinon treatments. NRR time was significantly decreased in adult earthworms exposed to diazinon (>64% reduction) and chlorpyrifos (>53% reduction). ChE activity and NRR assay resulted suitable biomarkers of pesticide suitable exposure, and it was linked to suitable changes in growth or fecundity.	(12)
E. fetida (adults)	Pb: lead acetate	Pb = 102, 221, 283, 588, and 1,233 mg kg^{-1} 28 d	Natural soils spiked with Pb Soil moisture = 20% Organic matter = 5.6% pH = 7.6 Temp. = 18°C 12 : 12 hr L : D cycle	NRR assay	NRR times showed a significant negative relationship with body Pb concentration. All treatments caused a reduction (>50% reduction) respect to the control group.	(13)

Table 1. *Continued*

Species	Test substance: chemical form	Concentration and time of exposure	Test conditions	Biomarkers	Main remarks	Reference[a]
A. caliginosa *E. fetida* *L. terrestris* *L. rubellus* (clitellate adults)	Zn: $Zn(NO_3)_2 \cdot 6H_2O$	Zn = 190, 350, 620, 1,200, 2,000, and 3,600 mg kg^{-1} 42 d	Natural soil (with 10% sphagnum peat) Soil moisture = 60% Temp. = 15°C Constant light	NRR assay	NRR assay reveals as a good biomarker for predicting adverse effects at life cycle level. NRR assay showed significant species-specific differences.	(14)
L. rubellus (clitellate adults)	Cu: $CuCl_2 \cdot 2H_2O$ Cd: $CdCl_2 \cdot 2\frac{1}{2}H_2O$	5, 25, 125, and 200 µg g^{-1} dry wt, each metal 21 d	Artificial soil (OECD) Soil moisture: 35%–40% pH = 6.0 ± 0.5 Temp. = 17°C 12 : 12 hr L : D cycle	wMT-1 wMT-2 AOX LYS	Exposure to Cu-spiked soils caused an induction of the biomarkers at the lowest Cu concentrations only. wMT-2 isoform showed a concentration–response relationship with increasing Cd concentrations in the soil. These molecular genetic biomarkers were more sensitive to Cu exposure than mortality, growth, or cocoon production.	(15)

AChE, acetylcholinesterase; MROD, methoxyresorufin-*O*-deethlylase; NADH Red, NADH cytochrome reductase; NADPH Red, NADPH cytochrome reductase; GR, glutathione reductase; GST, glutathione-*S*-transferase; LP, lipid peroxides; LPI, peroxidizable lipids; total GSH, total glutathione; % GSSG, percentage of oxidized glutathione; EROD, ethoxyresorufin-*O*-deethylase; ChE, cholinesterase; CYP1A, cytochrome P4501A isoenzyme; MT, metallothionein; NRR, neutral red retention; wMT-1, metallothionein isoform 1; wMT-2, metallothionein isoform 2; AOX, amine oxidase; LYS, lysosomal glycoprotein.

[a]References: (1) Ribera et al. 2001; (2) Saint-Denis et al. 2001; (3) Saint-Denis et al. 1999; (4) Rao et al. 2003; (5) Panda and Sahu 2004; (6) Brown et al. 2004; (7) Lukkari et al. 2004a; (8) Ricketts et al. 2004; (9) Svendsen and Weeks 1997a; (10) Scott-Fordsmand et al. 2000; (11) Reinecke and Reinecke 2003; (12) Booth and O'Halloran 2001; (13) Booth et al. 2003; (14) Spurgeon et al. 2000; (15) Burgos et al. 2005.

Earthworm biomarkers related to the detoxification systems have become of increasing concern. Using a similar exposure protocol, Ribera and coworkers examined the effects of Pb (Saint-Denis et al. 2001), carbaryl (Ribera et al. 2001), and benzo(*a*)pyrene (Saint-Denis et al. 1999) in a suite of biochemical biomarkers in *E. fetida andrei* (see Table 1). In general, a nonclear concentration–response relationship was observed for most of the biomarkers. However, factorial discriminant analysis of all biomarker responses enabled them to establish differences related to the toxicant concentration in soil. The use of multivariate statistics has been applied and suggested by others (Burgos et al. 2005) when concentration(dose)–response relationships are not clearly defined. The results by Ribera's group showed that the three assayed contaminants caused biomarker responses comparable to those found in other organisms such as fish (van der Oost et al. 2003). Thus, carbaryl drastically inhibited the acetylcholinesterase (AChE) activity, whereas Pb increased lipid peroxidation and caused inhibition of enzyme activities involved in xenobiotic metabolism such as glutathione-*S*-transferase (GST) or methoxyresorufin-*O*-deethylase (MROD). Similarly, benzo(a)pyrene caused an induction of the MROD and catalase activities and lipid peroxidation. The authors suggested that the formation of reactive oxygen species (ROS) accounts for the response of certain biomarkers such as catalase or GST or the increase in lipid peroxidation. The mechanism causing the lysosomal membrane fragility in earthworm coelomocytes is not yet well understood (Svendsen et al. 2004), although the participation of ROS should not be totally excluded. One of the effects of these highly reactive chemical species is the formation of lipid hydroperoxides from the polyunsaturated fatty acids, leading to altering membrane integrity and function (Abuja and Albertini 2001); this could be one of the mechanisms of toxic action leading to lysosomal membrane damage (Pellerin-Massicotte and Tremblay 2000).

Earthworms are important members in the agroecosystem because of their beneficial contribution to soil structure and function. Despite this, laboratory and field studies involving biomarkers for assessing pesticide impact on earthworms are still scarce in comparison to other organisms (Scott-Fordsmand and Weeks 2000). Organophosphorus (OP) and carbamates (CB), commonly named anticholinesterase (anti-ChE) pesticides, are an important group of agrochemicals widely used in modern agriculture. More than two decades of ecotoxicological research on ChEs have demonstrated that these enzymes are suitable biomarkers of pesticide exposure and toxic effects, and they continue to be an important component in the biomonitoring programs of pesticide contamination. In a standardized toxicity test (paper contact assay; OECD 1984), Rao et al. (2003) measured variations of AChE activity in *E. foetida* exposed to the median lethal concentration (LC_{50}) of chlorpyrifos. They found AChE inhibition above 60% after 12 hr exposure, which increased up to 91% after 48 hr OP exposure. Simultaneously, a gradual morphological damage in the animals (rupture of

the cuticle, bloody lesions, or fragmentation of posterior parts) was observed in relation to the chlorpyrifos concentration and time of exposure (24 or 48 hr).

Toxic effects of anti-ChE pesticides on the earthworm reproduction system have been described in *E. fetida*. In a histological study, Sorour and Larink (2001) showed that the fungicide benomyl caused gradual damage on the male reproduction system (abnormal cytophores and malformed spermatides) in individuals exposed for a week to sublethal concentrations (8.3–112 mg kg^{-1}). Likewise, Espinoza-Navarro and Bustos-Obregon (2005) also observed alterations in the male reproduction system in specimens exposed to the OP malathion (80–600 mg kg^{-1}). Besides a loss of body weight up to 50% in the treated groups compared to nonexposed, they also found vacuolization of spermatheca and fragmentation of DNA in a high proportion of spermatogonia. All these toxic effects probably cause alterations in the reproductive performance of earthworms. In this sense, the biomarker responses to this class of pesticides should be investigated in detail in future research. In their review, Scott-Fordsmand and Weeks (2000) showed that a considerable number of ChE-inhibiting pesticides have been assayed in earthworms but that the potential use of ChEs as biomarkers of pesticide exposure has not been sufficiently explored. For example, very few data exist on the recovery rate of phosphorylated or carbamylated ChE activity of earthworms. Indeed, one of the most important features in a good biomarker is the stability of its response, especially when it is used in the field. As an example, OP-inhibited ChE of birds take from hours to a few days for full recovery, whereas phosphorylated ChE in aquatic invertebrates, fish, or reptiles recovers its normal activity more slowly, taking several weeks for full recovery (Fulton and Key 2001; Sanchez-Hernandez 2001).

This slow recovery rate enables the detection of OP impact over a longer period after OP applications, a desirable feature when these types of pesticides show a low persistence in the environment (Racke 1992). Panda and Sahu (2004) determined the time to full recovery of AChE activity in the tropical earthworm *Drawida willsi* after exposure to butachlor (a herbicide), malathion, and carbofuran. Although butachlor did not cause any variation in AChE activity, maximum inhibition of AChE activity was found after 9 d exposure to malathion (2.2 and 4.4 mg kg^{-1}) and after 12 d exposure to carbofuran (1.1 and 2.2 mg kg^{-1}). The recovery of AChE activity of *D. willsi* was found to be extremely slow (45–75 d). Moreover, the recovery rate of the phosphorylated (or carbamylated) AChE activity did not appear to be related to the pesticide concentration. However, in that study earthworms were continuously exposed to the OP- or CB-contaminated soils, and it is difficult therefore to draw any conclusion about AChE recovery. To investigate the recovery rate of ChE activity, it would be ideal to transfer earthworms to clean soil when ChE activity is inhibited. This approach would be more environmentally realistic than keeping the earthworms

continuously in the contaminated soils for a long time, especially if earthworms tend to avoid contaminated soils (Schaefer 2003). Natural variability and impact of ambient variables on earthworm ChE activity need to be studied, as well as the ecological meaning of ChE inhibition (e.g., alterations of burrowing or feeding activities). On the other hand, there exist two main groups of esterases that participate in the manifestation of tolerance and resistance to ChE-inhibiting pesticides: fosfotriesterases and carboxylesterases (Jokanovic 2001; Sogorb and Vilanova 2002). To date, one study has reported the existence of fosfotriesterases in the earthworm *E. andrei*; these appear to be primarily localized in the intestinal tissues (Lee et al. 2001), but the implication in OP tolerance still needs to be explored.

Earthworms avoid contaminated soils. Several studies have demonstrated that the avoidance response of earthworms often occurs at low levels of metal concentration at which survival and reproduction are not affected (Schaefer 2005; Loureiro et al. 2005; Lukkari et al. 2005; Lukkari and Haimi 2005). van Gestel and Weeks (2004) reported that the earthworm behavior of avoiding contaminated soils should be among the aspects of earthworm ecotoxicology to be investigated. Indeed, there is a growing interest in the use of earthworm behavior in soil ERAs (Table 2). Different designs have been used for the avoidance behavior test. Schaefer (2003) compared test results from the most common test chambers, i.e., two- and six-chamber test systems. Although both systems gave similar results, the two-chamber system was recommended for future avoidance behavior tests mainly by its simplicity. This chamber consists of a rectangular container divided in two equal compartments by a removable plastic separator (Fig. 1). Control soil is placed in one compartment and the contaminated soil is placed in the other. A number of earthworms are then released in the middle of the rectangular container after removing the partition. The test starts when earthworms enter the soil, and 48 hr later, the partition is inserted again in the middle of the rectangular container. Individuals are counted in each soil compartment, and an avoidance response is judged as positive when more than 80% live earthworms is found in the compartment containing the control soil.

The two-chamber system is gaining acceptance in soil toxicology. Lukkari et al. (2005) used the avoidance test to examine whether the earthworm *Aporrectodea tuberculata* showed a positive response to Cu/Zn-contaminated soils. They exposed two natural populations of earthworms, with and without earlier wildlife exposure to metal-contaminated soils, to field soils spiked with seven Cu/Zn concentration pairings ranging from 23/41 to 267/467 mg kg^{-1}. Earthworms avoided the contaminated soils with Cu and Zn concentrations higher than 53 and 92 mg kg^{-1}, respectively. In this study, the avoidance response was a more-sensitive index than the standardized acute toxicity and reproductive tests. The avoidance behavior has also been applied to the toxicity assessment of field soils. Loureiro et al. (2005) tested soil samples collected from the abandoned mine Mina de Jales

Table 2. Avoidance Behavior Test in Earthworms Experimentally Exposed to Contaminated Soils.

Species	Test substance: chemical form	Concentrations	Test conditions	Test chamber	Main remarks	Reference[a]
Aporrectodea tuberculata (clitellate adults)	Cu: $CuCl_2 \cdot 2H_2O$ Zn: $ZnCl_2$	Cu/Zn = 23/41 to 267/467 mg kg^{-1}	Natural soil. Temp. = 18°C Soil moisture = 26%–27% pH = 6.0–5.8 Organic matter = 6.5%–7.5% Darkness 48-hr exposure	Cylindrical chamber (17 cm diameter × 11 cm depth)	Significant response (>80% individuals in control soils) was found in earthworms exposed to soils with Cu and Zn concentration pairings higher than 53 and 92 mg kg^{-1}, respectively. Soils contaminated with Cu and Zn concentration pairings higher than 79 and 138 mg kg^{-1}, respectively, caused an avoidance response in 80% of earthworms. Avoidance response test resulted to be a more sensitive endpoint than standardized toxicity tests (acute or reproduction tests).	(1)
Eisenia andrei (clitellate adults)	Carbendazim Benomyl Dimethoate Cu: $CuSO_4 \cdot 5H_2O$	Carbendazim and benomyl = 1, 10, and 100 mg kg^{-1} Dimethoate = 2.5, 5, 10, and 20 mg kg^{-1} Cu = 40, 80, 160, and 320 mg kg^{-1}	Artificial Lufa 2.2 soil Soil moisture = 60% pH = 5.03 Organic matter = 1.28% Temp. = 20° ± 2°C 16:8 hr L:D cycle 48-hr exposure	Rectangular chamber (21 × 12.3 cm)	Earthworms avoided the contaminated soils (>80% individuals in the control soil) at 320 mg kg^{-1} of Cu, >10 mg kg^{-1} of benomyl and carbendazim, and 40 mg kg^{-1} of dimethoate.	(2)
E. fetida (clitellate adults)	Total petroleum hydrocarbons (TPH) and 2,4,6-Trinitotoluene (TNT)	TNT = 2, 7, 29, and 1,142 mg kg^{-1}. TPH = 200, 316, and 1,047 mg kg^{-1}.	Natural contaminated soils (mixed with Lufa 2.2 soil to obtain different contaminant concentrations) Soil moisture = 60% pH = 6.0 ± 0.5 Temp. = 20° ± 2°C Darkness 48-hr exposure	Square chamber (20 × 20 × 10 cm)	Significant avoidance behaviour response (>90%) of the soil contaminated with 1,047 mg kg^{-1} of TPH and >29 mg kg^{-1} of TNT. Avoidance behavior response was a more sensitive endpoint than mortality.	(3)

Table 2. *Continued*

Species	Test substance: chemical form	Concentrations	Test conditions	Test chamber	Main remarks	Reference[a]
E. fetida (clitellate adults)	2,4,6-Trinitotoluene (TNT)	TNT = 2, 7, 29, and 1,142 mg kg^{-1}	Natural contaminated soils Soil moisture = 60% pH = 6.0 ± 0.5 Temp. = 20°C Darkness 48-hr exposure	Round container (28 cm diameter × 10 cm high) with six different chambers connected to a central chamber	Earthworm avoided the soils contaminated with TNT concentration higher than 29 mg kg^{-1}. Avoidance response of earthworm was a more sensitive indicator of TNT exposure than mortality or reproduction rate.	(4)
E. andrei, *L. rubellus*, and *A. caliginosa* (clitellate adults)	Pb: $Pb(NO_3)_2$	350–15,000 mg Pb kg^{-1}	Kettering loam soil. Soil moisture = 50% pH = 4.56 ± 0.01–5.84 ± 0.01 Temp. = 15°C (*A. caliginosa*) and 20°C (*E. andrei* and *L. rubellus*) Darkness 3-hr exposure	Petri dishes (210-mm diameter)	Avoidance response increased with Pb concentration for the three species. Almost all test individuals avoided the contaminated soil at Pb concentrations higher than 5,000 mg kg^{-1}. There was no significant difference between the avoidance behaviour of the three species.	(5)
Dendrobaena octaedra, *L. rubellus*, and *A. tuberculata* (clitellate adults)	Cu: $CuCl_2 \cdot 2H_2O$ Zn: $ZnCl_2$	Cu/Zn = 19/32 to 300/500 mg kg^{-1}	Field soil Temp. = 18°C Soil moisture = 27%–29% pH = 5.8–6.6 Organic matter = 13%–14% Darkness 48-hr exposure	Cylindrical chamber (17 cm diameter × 11 cm depth)	There was a species-specific response to the metal-contaminated soils. *D. octaedra* was the most sensitive species, whereas *L. rubellus* avoided the contaminated soil at the highest metal concentrations.	(6)

Table 2. *Continued*

Species	Test substance: chemical form	Concentrations	Test conditions	Test chamber	Main remarks	Reference[a]
E. fetida (clitellate adults)	Cu: $CuSO_4$, $Cu(NO_3)_2$, $Cu_2(OH)_2(CO_3)$	110–1,750 µg Cu g^{-1} using $Cu(NO_3)_2$ 600–5,000 µg Cu g^{-1} using $CuSO_4$ 1,100–17,500 µg Cu g^{-1} using $Cu_2(OH)_2(CO_3)$	Kettering loam soil. Soil moisture = 50% pH = 6.85 ± 0.05 Temp. = 20°C Darkness 24-hr exposure	Petri dishes (210-mm diameter)	Earthworms avoided contaminated soils at all the Cu concentrations assayed using $Cu(NO_3)_2$ and $CuSO_4$. No positive avoidance behaviour responses was observed when soil was spiked with $Cu_2(OH)_3(CO_3)$ concentrations ≤3,500 µg Cu g^{-1}. Avoidance behaviour response was more a sensitive index to assess Cu exposure than mortality or weight changes.	(7)
Aporrectodea nocturna and *Allolobophora icterica*	Imidacloprid: Confidor	1, 0.1, and 0.01 mg kg^{-1}	Field soil pH = 8.3 Temp. = 12° ± 1°C Organic matter = 28.3 g kg^{-1} Darkness 48-hr exposure	Plastic chambers (8-cm depth) 25 × 25 cm for *A. nocturna*, 15 × 25 cm for *A. icterica*	Both species avoided the contaminated soil at pesticide concentrations ≥0.1 mg kg^{-1}, being the avoidance response more evident for *A. icterica*.	(8)

References: (1) Lukkari et al. 2005; (2) Loureiro et al. 2005; (3) Schaefer 2003; (4) Schaefer 2005; (5) Langdon et al. 2005; (6) Lukkari and Haimi 2005; (7) Arnold et al. 2003; (8) Capowiez and Bérard (2006).

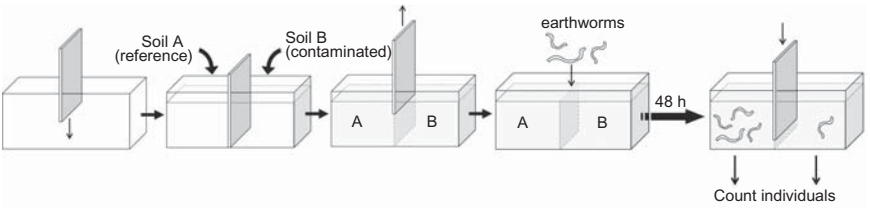

Fig. 1. Scheme of avoidance behavior response test.

(Portugal) with the avoidance response assay. The most contaminated soils (As = 251, Cd = 8.2, Cr = 15, Cu = 24, Mn = 255, Ni = 9, Pb = 209, Zn = 97 mg kg^{-1}) showed a significant behavior response in *E. andrei* when these soils were mixed (75% w/w) with an artificial control soil. In a similar study, Lukkari and Haimi (2005) exposed individuals of a natural earthworm population (*A. tuberculata*) to soils sampled close to a mining area in Finland. Metal-contaminated soils were first mixed with uncontaminated natural soil to obtain contaminated soil proportions of 25%, 50%, 75%, and 100%. Earthworms showed a significant avoidance response when they were exposed for 24 hr to soil containing 25% of the metal-contaminated soil. Although no biomarkers were involved in these studies, it would be attractive to establish a relationship between molecular biomarkers and avoidance behavior responses, especially when the earthworm behavior has direct ecological implications.

B. Field Studies

In a retrospective ERA, four types of approaches can be performed: (1) biological surveys, (2) laboratory tests of ambient media (e.g., soil, water, or sediment), (3) simulated field studies, and (4) *in situ* exposure bioassays. These approaches have used earthworms to assess toxicity of contaminated soils. Summarized next are those studies in which biomarkers were measured in combination with other toxicological endpoints (body residues, growth, survival, or reproduction rate).

Biological Surveys. A few studies have documented body contaminant residues and biomarker fluctuations in relation to soil contamination. Induction of the cytochrome P4501A (CYP1A) and GST activities and MT levels were examined in the earthworm *A. tuberculata* collected along a 4-km transect from an area contaminated by a steel smelter in Finland (Lukkari et al. 2004b). An increase in the response of the three biomarkers was positively correlated with decreasing distance from the steel smelter, which was accompanied by a progressive increase of metal concentrations in soils. Increase of MT levels and GST activity were not related to body metal concentration. Conversely, an induction of CYP1A,

measured by ethoxyresorufin-*O*-deethylase (EROD) activity, positively correlated with metal (Cu, Zn, Fe, and Al) concentrations in the earthworm tissues. Although CYP1A induction is generally attributed to organic contaminant exposure (Whyte et al. 2000), enzyme induction observed in *A. tuberculata* seemed due to metal exposure only. This unexpected finding was corroborated by the authors in a parallel laboratory experiment using natural populations of *A. tuberculata* exposed to a field soil spiked with Cu and Zn (Lukkari et al. 2004a; see Table 1). Laszczyca et al. (2004) also documented spatial and temporal variations of selected biomarkers (CbE, AChE, and antioxidant enzyme activities) in three natural earthworm populations (*A. caliginosa*, *L. terrestris*, and *E. fetida*) collected from meadow sites situated along a 32-km-long transect from a Zn/Pb ore mine and a smelter metallurgic complex. Although body metal (Zn, Pb, Cd, and Cu) concentrations increased in earthworms with decreasing distance from the point-source of pollution, biomarkers showed peak responses at the middle of the transect (4–8 km from the point-source of metal pollution). The authors attributed these biomarker responses to a hormetic-like effect, and suggested that this type of response could be useful in identifying areas where soil contaminants cause adverse effects on organisms in contrast to those areas where toxic effects are balanced by compensatory responses. Hormesis is defined as overcompensation to alterations of homeostasis (Chapman 2001). However, although hormesis is a phenomenon observed generally in the laboratory, its occurrence under field conditions is difficult to assess, mainly because many environmental factors can affect biomarker responses.

The high sensitivity of the NRR assay widely demonstrated in laboratory experiments has been also observed in field studies. A temporal study was carried out to assess the negative impact on the indigenous earthworm *Microchaetus* sp. of copper oxychloride applications (Maboeta et al. 2002). After simulated applications (at $4.25\,g\,L^{-1}$) of the fungicide on a demarcated area, earthworms were periodically sampled to complete a 6-mon survey, and NRR times were recorded. The NRR assay in *Microchaetus* sp. was a more sensitive indicator of pesticide exposure than earthworm biomass or abundance, a finding that agrees with the observations reported in laboratory experiments using other earthworm species and toxicants (see Table 1).

Laboratory Tests of the Soil. The biological survey approach presents a set of drawbacks such as lack of information about exposure history, difficulties in species identification and specimen collection in the sites of interest, the impact of environmental stressors other than the contaminants, and other sources of uncertainty. These limitations can be resolved, in part, when field soils are tested under stable laboratory conditions. The use of a model earthworm species (e.g., *E. fetida*) and controlled conditions (soil moisture,

pH, temperature, organic matter content, photoperiod, etc.) help to link biomarker responses to bioavailable contaminants in soil.

Similar to spiked soil experiments, the NRR assay has proved to be a highly sensitive biomarker of metal exposure when earthworms are exposed to field-contaminated soils. Scott-Fordsmand et al. (2000) found a significant relationship between NRR times measured in *E. fetida* and Cu concentrations in soils collected from a Cu-contaminated site in Denmark. Besides noting that the NRR assay was more sensitive to Cu exposure than reproduction rate, they found that field soils with 70 yr contamination history were less toxic than Cu-spiked soils. This observation suggests that results from standardized toxicity tests using spiked soils should be taken with serious reservations, and they should not be considered alone for decision making related to ecosystem management. In a similar study, Booth et al. (2003) exposed *E. fetida* to soils collected from prairie skeet ranges in Canada. The authors also found a rapid response of NRR assay compared to growth rate, cocoon production or cocoon viability. The highly significant correlations between NRR times and soil Pb concentrations, or concentrations of $Ca(NO_3)_2$-extractable Pb, demonstrated that the NRR assay can be a sensitive and predictive biomarker of earthworm Pb body burdens (or bioavailable Pb).

Simulated Field Studies. In general terms, these studies can be defined as artificially bounded systems that represent specific ecosystems or fractions of these. Their main application is to investigate the contaminant effects on organisms under the influence of multiple environmental fluctuating variables. Depending on the dimensions, it is possible to distinguish two types of artificial ecosystems: microcosm and mesocosm. A soil microcosm consisting of a cylinder (7.5 cm inside diameter × 15 cm high) made from high-density polypropylene pipe was used by Burrows and Edwards (2002) to assess the effects of the fungicide carbendazim on a representative group of soil organisms including plants, earthworms, and nematodes. This approach not only examines the toxic effects on each organism but also investigates the alterations on ecosystem processes such as nitrogen mineralization, nutrient transformation, or ecological interactions between organisms. Generally, soil microcosm experiments are carried out indoors under stable ambient conditions [temperature, light/dark (L : D) cycles, artificial rainwater, etc.].

An alternative man-made ecosystem segment of higher dimensions is the mesocosm, which is structurally and functionally closer to the "real world" than the microcosm. The mesocosm is generally constructed as an outdoor system, and environmental variables (pH, temperature, humidity, organic matter, etc.) are routinely recorded to help in the data interpretation. Mesocosms were employed by Svendsen and Weeks (1997b) and Spurgeon et al. (2005b) to study the effects of Cu and Cd on the earthworm *L. rubellus*;

they concluded that seasonal changes or fluctuating environmental conditions typical of northern temperate regions did not appear to affect significantly the toxicity of these heavy metals.

In Situ Exposure Bioassays. The least used approach in ERA, probably for logistic reasons, *in situ* exposure bioassays are generally performed in the site of interest when minimal alteration of soil (e.g., mix of horizons) and more realistic exposure conditions are required. An example of an *in situ* exposure assay is the study by Hankard et al. (2004), who used caged earthworms (*L. terrestris*) to assess the suitability of NRR assay and total immune activity (TIA) to soils contaminated by both heavy metals and the 16 priority pollutant PAHs. Although percent of survival was high, a significant reduction in the NRR time (<10 min) was found in earthworms caged for 12 d in the contaminated sites compared to NRR times (20–27 min) measured in worms deployed in the control sites. The TIA test was a less sensitive biomarker than the NRR assay after 12 d exposure. Exposure to heavy metals (Cu, Zn, Pb) and PAHs accounted for biomarker responses in *L. terrestris* because of the positive relationship found between the body residues and soil concentrations.

The main advantages and limitations of these four approaches of the retrospective ERA are summarized in Fig. 2. Factors such as the objectives of the ERA, the physical features of the site under study, the resources available for conducting the ERA, and the nature of the contamination are determinants in the selection of the best approach. Nevertheless, it is recommended to use more than one methodology integrated in a WOE framework to obtain a more reliable ERA of a contaminated site.

III. Discussion

A. Biomarkers in Standardized Toxicity Tests

In general, standardized toxicity tests are characterized by their simplicity, rapidity, and low cost. However, these attributes could lead to erroneous conclusions in environmental management decisions or bioremediation procedures. Four important aspects are frequently ignored when running toxicity testing, or when ecological consequences are forecast from the test results: (1) low contaminant concentrations in the field, (2) long-term exposure to sublethal concentrations of contaminants, (3) toxic effects from contaminant mixtures, and (4) fluctuating environmental factors affecting toxicity.

Intuitively, one would think that the levels of certain universal contaminants (e.g., organochlorine pesticides, polychlorinated biphenyls) in the environment have decreased in the past two decades due to measures such as the application of remediation technologies, improvement in the

	Laboratory test of field soils	*In situ* exposure bioassay
Advantages	Earthworms are exposed under stable conditions (e.g., soil temperature and moisture). Biomarker responses are easily related to the bioavailable fraction of soil contaminants. Low cost, rapid, and relatively low effort. Standardized protocols (e.g., OECD or ISO toxicity tests).	Influence of environmental variables on earthworm sensitivity to contaminants can be assessed. Homogeneous earthworms (e.g., weight, sexual maturation) can be used to minimize variations of biomarker responses due to biological variables. Impact of fluctuating environmental variables on biomarkers (natural variation of biomarker responses) can be assessed in the control (nonexposed) group. Possibility of assessing impact of earthworm activity (bioturbation) on contaminant distribution along the soil profile. Realistic exposure conditions. Earthworm movements are limited and exposure is better defined.
Limitations	Field soils are generally sieved <5 mm before testing, and the influence of soil structure on bioavailability and toxicity of contaminants are not considered. Lack of ecological meaning: impact of fluctuating environmental variables is not assessed. Risk for soil disturbation and contaminant alteration during transport and storage. Risk for erroneous conclusions related to ecosystem management based on a suitable (sensitive?) species. Soil conditions (moisture, temperature, pH) must be periodically controlled.	Vandalism and damage by adverse atmospheric conditions. Locating of suitable reference sites. Time consuming and relatively high effort (e.g., cage construction). Indigenous juvenile earthworms can be caged with sentinel earthworms if soil cores are not exhaustively examined before placed into the mesh bag. Biomarker responses highly influenced by environmental factors other than the contaminants. Effects from the deployment system.

Fig. 2. Advantages and limitations of ecological risk assessment (ERA) approaches used to assess toxic effects of contaminated soils.

	Ecological survey	Simulated field study(mesocosm)
Advantages	Indigenous earthworm population are sampled (ecological realism). Earthworms show integrated responses to accumulation and toxic effects. Relatively easy and low cost. Biomarker responses can be complemented by ecological index such as biomass or species richness. Sampling limited to wet seasons in temperate areas.	Used for assessing the impact of contaminated-spiked soils under natural conditions. Toxic effects and biomarker responses are environmentally realistic. Impact of fluctuating environmental variables on biomarkers can be assessed before add the contaminant (natural variation of biomarkers). Biomarker responses and toxic effects can be investigated in a considerable number of soil organisms other than earthworms. Indirect effects of contaminant can be investigated. Homogeneous earthworms (e.g., weight, sexual maturation) can be used. Earthworm movements are limited and exposure is better defined.
Limitations	Unknown exposure history, and tolerance or resistance to pollution could have developed. Difficulties in sampling and species identification. Biomarker responses are difficult to interpret (contaminant mixture effects, impact of biological variables). Relatively high costs of field surveys.	Time consuming and a relatively high effort is required. Vandalism and damage by adverse atmospheric conditions. Natural sieved soils are generally used, and extrapolation to field soil must be carefully performed.

Fig. 2. *(Continued)*

treatment of liquid or solid wastes, the forbidden manufacture and use of persistent organic pollutants in many countries, the use of low-persistent pesticides (OPs, CBs, or pyrethroids), and the shutdown of mining activities, among others. Under this hypothetical scenario of low contaminant levels, which is probably true for many environments suspected of being contaminated, the current purpose of ecotoxicity testing is questioned. Similar to sediment, soil is an environmental medium in which many types of contaminants accumulate up to concentrations potentially toxic to biota. A contaminant generally coexists with its metabolites, other types of contaminants, or different chemical forms (i.e., metal speciation). This chemical cocktail can be harmful to organisms as a result of synergism, potentiation, or antagonism interactions between toxicants. Eggen et al. (2004) stressed these aspects of the ecotoxicology and suggested focusing efforts on organism responses at molecular level (e.g., genomic and proteomic responses) using simple biological systems such as cells, subcellular systems, or unicellular organisms. However, predictions of adverse effects at population or community levels from molecular biomarker responses continue to be a challenge in ecotoxicology.

Appropriate biomarkers may be applied in standardized bioassays to provide evidence of the cause–effect relationship between soil contaminants and toxic effects in the individuals. In aquatic toxicity testing, the biomarker approach has brought about promising results. For example, Neuparth et al. (2005) included certain biomarkers (MT induction, DNA strand breakage, and lipid peroxidation) in a standardized sediment toxicity test to assess toxic effects at multiple biological organization levels. They found that several estuarine sediments affected the survival and reproduction of the amphipod *Gammarus locusta*. In addition, a positive response in the MT induction and the frequency of DNA strand breakage was found in the organisms, and they concluded that the use of biomarkers in these ecotoxicity tests can help to distinguish biological responses to contaminant exposure from those originating from physicochemical variables of the sediment.

Biomarkers have also been applied in standardized toxicity tests using earthworms. Most of these studies have tried to link biomarker responses to adverse effects on life cycle traits. Ideally, the biomarker should show a concentration-dependent response to pollutants, particularly under stable experimental conditions. However, many laboratory studies involving earthworm biomarkers do not show a straightforward dose–response relationship (see Table 1). For example, many studies have reported that the NRR response linearly correlates with heavy metal concentrations in soil, or its bioavailability fraction, as well as with the metal body burden. However, this consistent cause–effect relationship needs to be validated for other classes of contaminants (PAHs, pesticides, PCBs, Hg) before making conclusions about its potential as predictor of deleterious effects at individual or population levels.

B. Toward an Environmentally Realistic Assessment of Contaminated Soils

Environmental processes influencing the contaminant toxicity in the nature are difficult to replicate in the laboratory. Consequently, a direct relationship between laboratory toxicity test results and ecological consequences could be extremely risky. Although this statement is well accepted by most ecotoxicologists, the results from standardized toxicity tests are generally used to identify a chemical as slightly or highly toxic and for environmental management decisions.

In general, soil toxicity tests with earthworms are typically performed using the OECD artificial soil or the LUFA 2.2 standard soil (see Jänsch et al. 2005 for soil characteristics). The general procedure involves the mixture of the test substance, using aqueous solutions for heavy metals or solvent solutions in the case of hydrophobic organic contaminants, with the artificial soil. After a few days of equilibration, earthworms are released into the spiked soils and the test is started. A more environmental realistic approach is to perform laboratory toxicity tests with field soils (see Fig. 2). Nevertheless, some limitations of these standard procedures should be taken into account. In a comparative context, Chapman et al. (2002) examined the ecological meaning of sediment toxicity tests and provided a number of issues and recommendations to be considered in future sediment ERA. Some of them are cited here to compare with soil toxicity testing:

The test organism is generally a species relatively easy to culture under laboratory conditions; however, it is sometimes more resistant than the native related organism.

The test organism is not often the best species for assessing toxicity or bioaccumulation in sediment toxicity testing. For example, the amphipod *Hyalella azteca* is a common organism in sediment toxicology. However, the natural behavior and food habits of this aquatic species are not simulated in the test chambers used in the standardized tests. The use of this species as a suitable test organism is thereby questioned (Wang et al. 2004).

The most common endpoints in sediment toxicity assays are survival, reproduction, and immobilization. Sometimes these do not define the potential toxicity of sediment contaminants, and the biomarker approach may be an important line of evidence (Neuparth et al. 2005; Costa et al. 2005).

Natural populations can develop tolerance or resistance to pollution by acclimatization or genetic selection. These aspects should be considered in ecotoxicity testing with native organisms (Chapman et al. 2002).

In addition, one of the main problems in sediment toxicity tests is the alteration of the sample during collection, handling, and storage, which can chemically transform the contaminants and consequently their bioavailability and toxicity (Ingersoll 2003). In view of these limitations existing in

sediment toxicology, should we consider related issues, and others, when running toxicity tests using field or contaminant-spiked soils? A large base of evidence suggests that some modifications should be included in the current soil toxicity protocols.

The Test Earthworm. For decades, standardized soil toxicity tests have been carried out using primarily two earthworm species: *E. fetida* and *E. andrei*, which were regarded as one species, termed indiscriminately as *E. fetida* or *E. foetida*. Currently, *E. fetida* and *E. andrei* are two different species (Dominguez et al. 2005) with differences in biological features (growth rate or cocoon production) of ecotoxicological concern (Jänsch et al. 2005). As for *H. azteca*, the ecological relevance of using these two species is also under current discussion. Biological and ecological aspects of these two *Eisenia* species, as well as the toxic effects of many classes of chemicals, are well known. Therefore, their use in soil toxicity testing could be justified. However, exposure of these species to soil contaminants is sometimes questioned, mainly because of the natural habits of these earthworms. *E. fetida* and *E. andrei* are epigeic earthworms that live in the soil surface, forming no permanent burrows, and feed on decaying organic matter. They require a high content of organic matter in soil (Jänsch et al. 2005), which explains why they are commonly found in compost heaps, manure piles, or sewage sludge. The question could be: Are these species suitable bioindicators when contaminants occur at soil depths where these earthworms are rarely found? When deep soil layers are tested for toxicity, are the test results ecologically realistic when using *Eisenia*?

Again, the example of the amphipod *H. azteca* examined in Wang et al. (2004) is useful to call into question the use of an inappropriate organism to extrapolate laboratory results to the field. In nature, this aquatic invertebrate is always found grazing on macrophytes, and contact with sediment is minimal or nonexistent; however, it is used for assessing sediment toxicity. Standardized test guidelines force *H. azteca* to burrow into sediment because assays are generally run without macrophytes and under constant light or L:D cycles (*H. azteca* is negatively phototactic). Laboratory soil testing conditions with *Eisenia* generally involve continuous light to force the earthworms to stay in the soil throughout the test (see Table 1). However, *E. fetida* and *E. andrei* are litter dwellers on the soil surface and generally do not ingest large amount of soils. Despite this, *Eisenia* is compelled to behave like an endogeic earthworm during the test. It is likely that we are making the same experimental error with *Eisenia* in soil toxicity testing as for *H. azteca* (Wang et al. 2004). From the ecotoxicological aspect, it would be desirable to use indigenous nonexposed earthworms as test organisms to achieve ecological realism.

Metal speciation is a determinant factor in the bioavailability of the heavy metals, which is, in turn, highly dependent on physicochemical features of the soil (e.g., pH, moisture, and organic matter). However, recent

studies have demonstrated that earthworms are able to alter the chemical forms of the metals in soil. Wen et al. (2004) found significant variations in heavy metal concentrations in three fractions extracted according to the Community Bureau of Reference's protocol (1, water soluble, exchangeable, and carbonate bound; 2, Fe- and Mn oxides bound; and 3, organic matter and sulfide bound). After incubation of soils in the presence of *E. fetida*, the metal concentrations associated with the bioavailability fraction increased. Changes in metal availability seem to be dependent on earthworm habits. In a laboratory experiment, Zorn et al. (2005a) found that the epigeic earthworm *L. terrestris* contributed to the increased availability of Zn ($CaCl_2$-exchangeable Zn) after 80 d. In contrast, the endogeic earthworm *A. caliginosa* was able to decrease Zn availability after 175 d (Zorn et al. 2005b). Modification of metal availability by earthworm activity is a matter of increasing concern in earthworm ecotoxicology and could have a notable application in the phytoremediation of contaminated soils.

The Test Substance. Davies et al. (2003a) demonstrated that the chemical form of the test substance significantly affects the test results. They exposed *E. fetida* to three chemical forms of Pb [$Pb(NO_3)_2$, $PbCO_3$, and PbS] following the OECD guideline for acute and chronic toxicity testing. In their experiments, the solid salts were added directly to the soil to attain the desirable Pb concentrations. The results revealed differences in cocoon production in relation to the chemical form of Pb. Toxic effects of Pb salts were related to their water solubility; the most toxic Pb salt was the most water soluble, i.e., $Pb(NO_3)_2$. This result could be explained because Pb uptake (dermal and gut exposure) requires the metal to be in solution. Similar results were obtained by Arnold et al. (2003), who exposed *E. fetida* to both aqueous and solid forms of several Cu salts [$CuSO_4$, $Cu(NO_3)_2$ and $Cu_2(OH_2)CO_3$]. The more water-soluble salt, i.e., $Cu(NO_3)_2$, was the most toxic Cu form. In addition, they found that the form in which Cu was applied to soils (aqueous or solid) did not significantly affect the results of acute and sublethal tests as well as the avoidance behavior response.

In these two related studies, it was also demonstrated that the conventional extractable procedures for measuring the metal fraction available to plants (water, $CaCl_2$, or diethylenetriaminepentacetic acid) are indicative of low metal availability to earthworms. As an alternative approach, the use of selected biomarkers (e.g., inhibition of delta-aminolevulinic acid dehydratase (ALAD) activity, MT induction) together to metal body burdens could help to determine the bioavailable, and bioactive, fraction of the metal. For example, highly significant correlations have been reported between the response of the NRR assay and the body Pb concentrations (Reinecke and Reinecke 2003).

The study by Davies et al. (2003b) also demonstrated that the bioavailability of Pb, added to test substrate as a $Pb(NO_3)_2$ solution, decreased over time probably because Pb did not rapidly reach equilibrium with soil. In fact, acute toxicity was higher when earthworms were immediately released

after soil spiking with $Pb(NO_3)_2$ than when added after soil–Pb equilibrium. As suggested by the authors, the equilibrium concept between soil and metal has serious implications in the laboratory-to-field extrapolations. A field study by Scott-Fordsmand et al. (2000) also illustrates the importance of considering the contamination history of the soil. They collected soils in a metal-polluted area with more than 70 yr Cu contamination. These soils were less toxic to *E. fetida* than soils spiked with the chloride salt of Cu. They concluded that differences for Cu toxicity could be explained by variations in Cu speciation, a result of Cu equilibration with soil. The time for equilibrium depends on the toxic substance and soil type. In a speculative context, the equilibrium phase for phophorothioate types of OP insecticides could lead to an increase of their toxicity because these compounds need to be transformed to the highly toxic "oxon" form by soil microorganisms or physicochemical factors, but simultaneously OP degradation can also occur. Thus, the time for equilibrium between soil and OP pesticides can be a critical parameter in ecotoxicity tests.

The Exposure Conditions. In a conventional acute or chronic toxicity test with earthworms, factors such as temperature, soil moisture, or photoperiod are kept at stable optimal values so that the only stress factor is the test substance or the contaminant mixture in the field soil. Obviously, this is not the only stress factor in the field, and many fluctuating environmental variables contribute to change earthworm sensitivity to pollutants (van Straalen 2003). One study shows clearly how toxicity is strongly influenced by environmental variables and therefore should be considered in future toxicity testing schemes. Bindesbøl et al. (2005) exposed the freeze-tolerant earthworm *Dendrobaena octaedra* to a range of Cu concentrations and different temperature regimens to investigate possible interactions between these two stress factors. Two important findings were reported: (i) acute Cu toxicity was affected by ambient temperature and metal toxicity increased with decreasing temperature, and (ii) there was a negative relationship between frost tolerance of the earthworm and Cu concentration in soil. In a comparative study, Spurgeon et al. (2005b) evaluated the impact of environmental factors on Cd and Cu toxicities in both adult and juvenile specimens of *L. rubellus* exposed to the metals for 70 d using a mesocosm system. Results were then compared with analogous experiments carried out under laboratory conditions (Spurgeon et al. 2003, 2004). They found no substantial differences in biomarker responses (metal-binding protein MT-2 or NRR assay) or life cycle traits (survival, growth, and reproduction) between those exposed in the mesocosm and those exposed under laboratory conditions. It was concluded that climatic conditions such as temperature (ranging from 15°–20°C to 5°C) or soil moisture (rainfall up to 20–25 mm resulting waterlogging) did not alter the sensitivity of *L. rubellus* to Cu or Cd. The results of this study and those by Bindesbøl et al. (2005) seem to draw contradictory conclusions, which encourages future investigations

aimed to demonstrate if fluctuating environmental variables such as temperature, soil moisture, pH, or organic matter content have a significant influence on earthworm sensitivity to pollutants.

In aquatic toxicology, *in situ* exposure using caging systems has gained acceptance, and a more realistic picture about ecological consequences from sediment contamination is often obtained. Suitable organisms can be exposed to water column, surface sediment, or sediment pore water using appropriate caging systems (Burton et al. 2005). Surprisingly, caged earthworms have rarely been used for assessing soil toxicity *in situ*. Several phenomena are not generally replicated in the laboratory, mostly for logistic reasons. For example, it has been demonstrated that some epigeic (*L. terrestris*) and endogeic (*A. caliginosa*) earthworms are able to transport contaminated soil from deeper layers to the soil surface, contributing to increased risk of adverse effects to other surface soil organisms. Thus, *in situ* exposure bioassays become a suitable approach for investigating the impact of this bioturbation process on soil toxicity.

C. Biomonitoring the Effectiveness of Bioremediation and Agrienvironment Schemes

Mining is among the main human activities causing metal pollution of soils. Although many mines have stopped their activity in numerous countries, they have contributed to greatly increased metal concentrations in soils. As an example, the Almadén mining district in Central Spain is one of the largest mercury mineral deposits in the world (Rytuba 2003), and it has been intensively mined since Roman times. A hazardous legacy was left inevitably: it is one of the most Hg-contaminated places on the Earth (Higueras et al. 2006). Here, although mining activity has ceased entirely, Hg concentrations up to 8,890 $mg\,kg^{-1}$ dw are commonly measured in soil. In an attempt to recover these heavily contaminated sites, a number of remediation processes have been, and continue to be, developed. Among them, phytoremediation, i.e., use of plants for environmental restoration, is of particular concern because heavy metals cannot be degraded, and their removal by plants seems to be an effective and environmentally friendly method (Lasat 2002).

One of the main limitations of phytoremediation is metal bioavailability. It has been demonstrated that earthworms are able to increase metal uptake by plants, thereby increasing the efficiency of phytoextraction (Wen et al. 2004). This beneficial "cooperation" has also been used to recover contaminated soils containing harmful organic chemicals such as PCBs or petroleum hydrocarbons; however, in these cases plants are substituted by microorganisms. Singer et al. (2001) used the anecic earthworm *Pheretima hawayana* to increase the degradation rate of arochlor 1242 by the bacteria *Rhodococcus* sp. ACS and *Ralstonia eutrophus* H850. In a similar study, Schaefer et al. (2005) investigated the effects of three species (*E. fetida, A.*

chlorotica, and *L. terrestris*) on soils spiked with petroleum hydrocarbons [10,000 mg kg^{-1} total petroleum hydrocarbons (TPHs)]. The authors concluded that earthworms increased the degradation rate of hydrocarbons after 28 d incubation, probably as a result of stimulation of microbial activity. Furthermore, such an increase in TPH degradation was species dependent with the following order: *L. terrestris* (30%–42% TPH decrease) > *E. fetida* (31%–37%) > *A. chlorotica* (17%–18%).

On the other hand, earthworms have been used to assess the effectiveness of soil bioremediation procedures. In a laboratory experiment, Morgan et al. (2002) determined body metal concentrations in the earthworm *L. rubellus* after 4 wk exposure to metal-contaminated soils that were previously treated with several chemical ameliorants (montmorillonite, hydroxylapatite, or ferrous oxide). They concluded that the use of earthworms as sentinel species can be a suitable approach for screening remediation effectiveness. In a related study, Davies et al. (2002) evaluated the efficacy of bone meal (phosphorus source) treatment in Pb-contaminated soils through ecotoxicological tests using *E. fetida*. Treatment of soils with bone meal (1:20) resulted in an increase of earthworm survival (7, 14, and 28 d exposure), growth, and cocoon production, and a decrease of Pb bioavailability. Lock and Janssen (2003) used adults of *E. fetida*, among other soil invertebrates, to determine the capacity of metal-immobilizing agents (called by the authors type I and type II) to reduce bioavailabity of Zn in contaminated soils from Belgium. The addition of these agents (5% w/w) to the soils, allowing 1 yr for equilibration before starting toxicity testing, resulted in a total elimination of soil acute toxicity (100% survival of *E. fetida* after 21 d exposure). The effectiveness of chemical immobilization amendments to metal-contaminated soils was also assessed through a 14-d toxicity test using *E. fetida* following the American Society for Testing Materials (ASTM) guideline (Conder et al. 2001). Toxicity of metal-contaminated smelter soils was significantly reduced when soils were treated with municipal sewage sludge biosolids stabilized with lime.

In these bioremediation studies, determination of biomarkers was not included despite that they are an indirect biological measure of contaminant available and toxic fraction. On the other hand, conclusions about remediation effectiveness are based on acute toxicity test results using a single earthworm species (*E. fetida*), which is not necessarily the most sensitive. In addition, acute bioassays do not show sublethal toxic effects, and chronic bioassays are required to provide long-term ecological impacts from contamination. Monitoring methods for assessing the progress of remediation actions in contaminated soils are traditionally based in chemical analysis of soil, employing sophisticated and high-cost instrumental analysis. Maila and Cloete (2005) reviewed the biomonitoring tools most used for evaluating effectiveness of the bioremediation for restoration of hydrocarbon-contaminated soils. Soil enzyme activities (lipase, dehydrogenase, urease, catalase), microbial biomass, microbial bioluminescence, seed

germination, and earthworm survival tests are among the main biological indicators for assessing soil remediation procedures. Maila and Cloete (2005), in line with other authors, concluded that it is necessary to develop new biomonitoring methods of soil remediation based on the use of ecologically relevant species. Biomarkers were not mentioned among these recommendations. In light of the literature discussed in this review, it can be concluded that certain earthworm biomarkers, such as the NRR assay or the avoidance behavioral response, can be useful indicators of sublethal effects during a soil remediation procedure.

Currently, it is widely accepted that modern agriculture represents a serious threat to wildlife. In the European Union, the increasing concern in developing environmentally-friendly agriculture has led to the introduction of the agrienvironment schemes (AES) in many Member States (Council Regulation No. EEC 2078/92). Reduction of fertilizer and pesticide inputs are among the most important measures. However, there exists a lack of information about the real effectiveness of European AES. An exhaustive review examined the most relevant ecological studies on the efficacy of the AES, measured in terms of changes in biodiversity (Kleijn and Sutherland 2003). It was concluded that the implementation of these schemes increased the biodiversity of several zoological groups such as insects or birds. Hole et al. (2005) also reviewed a considerable number of studies that compared the impacts of organic (no use of synthetic chemicals) and modern farming systems on biodiversity. From the 76 studies reviewed, only 13 involved comparisons of earthworm abundance and activity between organic and modern agriculture. In line with the results for other taxa (birds, soil microbes, spiders, butterflies, and others), most of the earthworm studies indicated that organic farming contributed to a higher abundance and species richness of earthworms compared to modern agriculture fields.

Kleijn and Sutherland (2003), however, called into question the use of comparative biodiversity studies between AES-implemented fields and control areas (modern agriculture) to assess the success of these schemes. They suggested that ecological evaluations must be initiated at the time that AES are implemented, comparing control and AES spots randomly selected in the same study area where AES began to be introduced. Therefore, this approach would allow a more reliable assessment of the effectiveness of AES.

Most of the investigations on the AES effectiveness are based in abundance and/or species richness studies. Unquestionably, these studies respond to one of the objectives of the schemes: protection of biodiversity. However, short-time responses to the AES introduction can be required in many cases so that remedial procedures can be included in time. The abusive use of pesticides and fertilizers in modern agriculture is a current practice that the AES implementation tries to reduce. In general, current insecticides and herbicides have a low persistence in the environment; however, many of them show high acute toxicity (OP and CB), which could

justify the inclusion of biomarkers in future biological surveys of pesticide applications. The use of *in situ* exposure bioassays using earthworms in the agricultural field with and without AES implementation could be a complementary approach to assess the impact of AES in the agrienvironment with consequence for the local earthworm biodiversity (Sepp et al. 2005).

IV. Perspectives in Earthworm Biomarkers

Most biomarkers provide an indication of pollutant exposure only. Under this consideration, the general strategy is to assay a suite of biomarkers covering molecular to whole-organism endpoints to obtain clear evidence of individual health deterioration (Beliaeff and Burgeot 2002; Handy et al. 2003). In the past 5 years, significant progress has been achieved regarding certain earthworm biomarkers such as MT induction or the NRR assay. In addition, new and promising biomarkers have been explored such as the induction of annetocin, a neuropeptide involved in the induction of egg-laying behavior in earthworms (Ricketts et al. 2004). Traditionally, earthworms have been used as bioindicators of metal pollution. Thus, biomarkers related to metal exposure (MT induction) have been extensively investigated (Kammenga et al. 2000; Scott-Fordsmand and Weeks 2000; Burgos et al. 2005). Other biomarkers (e.g., ChE, CbEs, or CYP1A) commonly used in biomonitoring programs with vertebrates have received little attention in earthworm studies. These organisms are considered suitable indicators of environmental change in agricultural environments (Paoletti 1999). Paradoxically, very few studies have involved the impact of pesticides on earthworms through the use of biomarkers of pesticide exposure. For example, earthworm ChE activity is sensitive to OP or CB pesticide contamination, and a slow recovery rate is frequently observed after ChE inhibition (Booth and O'Halloran 2001; Panda and Sahu 2004). However, the use of this well-known biomarker under field conditions has scarcely been investigated. Moreover, measurements of earthworm ChE activity levels together with the chemical reactivation of the enzyme in the presence of pralidoxime (McInnes et al. 1996; Sanchez-Hernandez 2003) could be a suitable methodology for identifying exposure to OP and CB pesticides in field.

Behavioral responses are included in the biomarker definition by several authors (Lagadic et al. 2000; Walker et al. 2001); nevertheless, they have had low consideration in ecotoxicological research compared molecular biomarkers. The behavior of an organism is defined as the final integrated result of a diversity of physiological processes interacting with the abiotic and biotic components of the environment (Fig. 3). Sensory, hormonal, neurological, and metabolic systems are the main physiological systems involved in behavior performance, and in turn, they represent the primary target systems of many contaminants.

Behavioral responses to pollution are becoming a matter of increasing concern in ecotoxicology. A substantial volume of literature describing

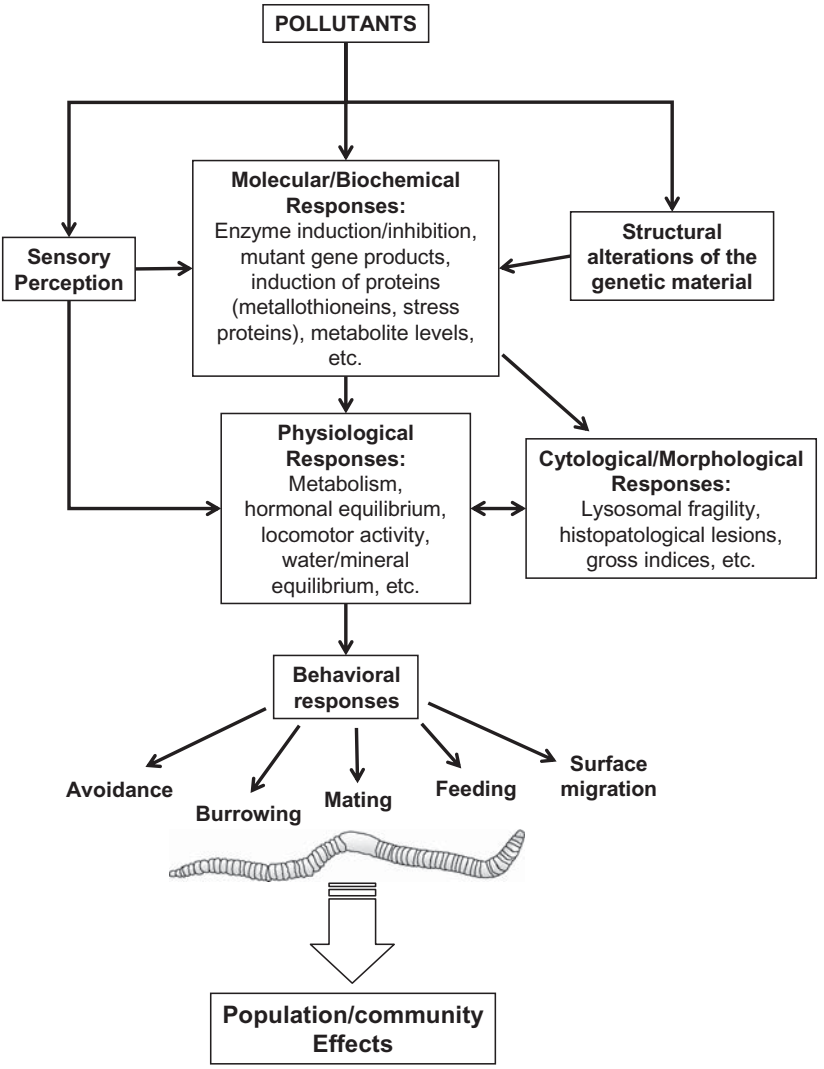

Fig. 3. Scheme of earthworm biological responses to pollutants. Behavioral changes are the result of the integration of several physiological systems affected by pollutants.

perturbation or disruption of physiological systems directly involved in fish behavior has been reviewed by Scott and Sloman (2004). According to the concept of a hierarchical cascade of biological responses to pollution occurring at different levels of biological organization, behavioral responses could be the key biomarkers for making predictive assessments of pollution at population or community levels. Efforts to correlate molecular

biomarkers to behavioral changes, with direct ecological implications, could be one of the future challenges in earthworm ecotoxicology. A well-known example is the relationship between brain AChE inhibition by OP/CB insecticides and behavioral disturbances in vertebrates (Peakall 1992; Sanchez-Hernandez 2001; Hill 2003; Bain et al. 2004). However, the absence of studies on disturbance of earthworm behavior by pesticides does not permit drawing any conclusions about this well-established relationship. Capowiez et al. (2003) examined the response of two common biochemical biomarkers (AChE and GST) and the burrowing behavior of two earthworm species exposed to the chloronicotinyl insecticide imidacloprid. Burrowing behavior was a more sensitive endpoint than biochemical biomarkers, which did not respond to the insecticide. However, behavior is the final product of many interacting physiological systems, and pollutants can interact with many of these systems. Thus, the identification of involved biochemical biomarkers becomes a difficult task.

Earthworm biomarkers need still to be investigated extensively to use them for predictive assessments of ecological consequences from pollution. In line with the main recommendations from van Gestel and Weeks (2004), it is opportune to add other lines of future research:

Biomarkers are sensitive indicators of exposure and should be included in the standardized toxicity tests under a well-developed and defined WOE framework. Biomarkers will make a significant contribution in acute bioassays as a measurement of the bioavailable and bioactive fraction of contaminants and in chronic bioassays as sublethal endpoints. The promising results obtained in sediment toxicology (Neuparth et al. 2005) encourage the application of biomarkers in soil bioassays.

The knowledge gained on certain earthworm biomarkers such as the MTs or the lysosomal membrane stability stimulates the development of standardized earthworm biomarker assays. This is an important step in applying biomarkers in a regulatory context. However, international agreement for developing a standard operating procedure for biomarker determination could become a difficult task with several biomarkers such as MTs, which can be measured by multiple analytical techniques (e.g., spectrophotometric, chromatographic, polarographic, or inmunodetection assays).

The main ecotoxicological meaning of the biomarker approach is to make predictions on changes in populations or communities from subcellular or individual responses. However, very little research has demonstrated such a relationship. Biochemical or physiological biomarkers could have an ecological meaning when they can be related directly to behavioral responses with significant ecological impact. The most common behavior response measured in earthworms is the avoidance of contaminated soil. However, Capowiez and Bérard (2006) pointed out that "avoidance is not a measure of toxicity but rather a measure of repellence". In

agreement with this assumption, the impact of contaminants on other behavioral responses such as burrowing, feeding, or surface migration must be studied together as biomarker responses.

To date, most of the earthworm biomarker investigations have been performed in a heavy metal pollution scenario. There is a need for increasing the knowledge of biomarkers of exposure to organic contaminants of current concern, i.e., anti-ChE insecticides, pyrethroids, brominated flame retardants, and PAHs. Biomarkers related to insecticide toxicity (e.g., AChEs) and detoxification (CbEs, phosphotriesterases, or CYP450-dependent monooxygenases), or biomarkers of oxidative stress requires further exploration to obtain a better understanding of the negative impact of organic pollutants on earthworms.

New biomarkers need to be investigated, especially when they could be directly involved in earthworm survival. For example, Na^+/K^+-ATPase is an important electrogenic component in the contraction mechanism of longitudinal muscle fibres of *L. terrestris* (Volkov et al. 2000), and it has been demonstrated in fish and aquatic invertebrates that this adenosine triphosphatase is inhibited by a wide range of heavy metals and pesticides leading to osmoregulation impairment.

Summary

Earthworms have had a notable contribution in terrestrial ecotoxicology. They have been broadly used to assess environmental impact from metal pollution, and they are typical test organisms (e.g., *Eisenia*) in standardized toxicity tests. Several reviews and international workshops have stressed the need for increasing the understanding and applicability of earthworm biomarkers in the ecological risk assessment (ERA) process. This review summarizes recent available information concerning the most investigated earthworm biomarkers. In earthworms, the use of biomarkers has been focused on assessing metal pollution, and available data on biomarker responses to organic contaminants are rather limited. The potential for applying earthworm biomarkers in the standardized toxicity tests is suggested in view of their significant contribution to the risk assessment of contaminated soils (e.g., estimation of bioavailable and bioactive fraction or sublethal effects). Field studies involving earthworm biomarkers are still scarce and are summarized according to their main practical approaches in retrospective ERA: biological surveys, laboratory tests of the soil, simulated field studies, and *in situ* exposure bioassays.

Despite the great volume of laboratory studies on earthworm biomarkers, future lines of research are suggested besides the recommendations made by others: (1) the potential and limitations of the inclusion of biomarkers in the standardized toxicity tests should be examined under a well-defined weight-of-evidence framework; (2) it is necessary to develop operating guidelines to standardize earthworm biomarker assays, an impor-

tant step to apply biomarkers in a regulatory context; (3) molecular and physiological biomarkers should be directly linked to behavioral changes with significant ecological implications, an important step in considering them as ecotoxicological biomarkers; and (4) biomarkers to organic pollutants of current concern (e.g., polycyclic aromatic hydrocarbons, anti-ChE and pyrethroid insecticides, polybrominated flame retardants, etc.) need to be developed and validated in the field. Also, an increase in the knowledge of earthworm biomarkers is undoubtedly useful in assessing the effectiveness of procedures for recovering/protecting the environment (e.g., phytoremediation or agrienvironment schemes) besides its potential use in the ERA framework.

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