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Review

Earthworm biomarkers of pesticide contamination: Current status and perspectives

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Earthworms are standard test organisms in soil toxicity testing. They have been broadly used to assess environmental impact from heavy metal pollution; however, the knowledge on toxic effects from pesticides upon these organisms is still very limited. One of the ecotoxicological approaches to assess pollutant bioavailability and sublethal effects is the use of molecular and biochemical biomarkers. This review focuses on five issues that need further investigation: 1) field validation of earthworm biomarkers of pesticide exposure (*e.g.*, cholinesterases) as well as testing and development in earthworms of those biomarkers of pesticide exposure currently used in other organisms (*e.g.*, carboxylesterases), 2) the impact of environmental and biological interfering factors upon biomarker responses, 3) the development of biomarker-based approaches to assess long-term pesticide exposure, and 4) the need to develop biomarkers of behavioural and reproductive disruption with direct implications at individual and population levels.

Keywords: earthworm, pesticide, biomarker, behavioural toxicology, long-term exposure.

Introduction

Earthworm activity has a notable influence on soil properties that contribute to increased soil fertility and plant performance, such as pH, texture, porosity and organic matter content.[1)] These annelids have a set of ecological and physiological features that make them excellent indicators of soil pollution compared to other terrestrial invertebrates.[2,3)] Earthworms are found in a wide variety of soil types and horizons, thereby being classified in three ecological categories: the epigeic species that are surface active and dwell in litter (*e.g.*, *Eisenia fetida*), the endogeic species that live in the organic horizons and create horizontal burrows (*e.g.*, *Aporrectodea caliginosa*), and the anecic species that live in vertical and deep burrows and ingest large amounts of soil (*e.g.*, *Lumbricus terrestris*).[4)] Earthworms are therefore continuously exposed to soil contaminants through their exterior epidermis and alimentary surfaces. Many species are large size (*e.g.*, anecic species) and the measurement of pollutant residues and biomarkers is feasible in a single individual as well as in different tissues or organs. Earthworms are also the common prey of many vertebrate species such as birds and shrews; they play therefore a key role in the biomagnification process of several soil pollutants and in the occurrence of indirect effects on terrestrial vertebrates from soil pollution.[5)] These characteristics, and others, have made earthworm one of the most common standard organisms in soil toxicity testing.[6)] They also are excellent bioindicators in the field monitoring of soil pollution. Thus, changes in earthworm abundance or species richness have been positively correlated to point-sources of soil pollution or to the level of soil degradation by agricultural activity.[4,7)] In addition, these organisms are suitable indicators for monitoring the effectiveness of polluted-soil remediation procedures.[8)] Nevertheless, the assessment of heavy metal pollution has been the main scope in most of the ecotoxicological investigations with earthworms,[9)] and very few studies have examined the toxic effects upon earthworms from currently used organic pollutants such as pesticides.

Biomarkers are an important element in the ecological risk assessment of pollution; they are used to estimate the exposure level and sublethal effects from pollutants. Biomarkers are commonly defined as measurable biological changes, from molecular to behavioural levels, in response to one or more contaminants.[10,11)] This approach has been well developed in aquatic (eco)toxicology,[12,13)] and sediment toxicity testing usually includes biomarker measurements that give solid evidence for pollutant bioavailability, and identify the

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main classes of pollutants involved in toxicity responses.[14] However, earthworm biomarkers have been scarcely investigated even though these animals are the standard test organism in soil toxicity testing.

It is not the purpose of this review to make a full compilation of studies on earthworm biomarkers as several monographs have extensively examined the knowledge on this subject.[15–17] This paper solely focuses on the advances in earthworm molecular and biochemical biomarkers of pesticide exposure. Comparisons with related biomarker studies in other organisms will allow identification of the main gaps in the knowledge of earthworm biomarkers and will put into perspective the need for a greater understanding of the following issues: 1) field validation of biomarkers of pesticide exposure currently used in earthworms and testing of biomarkers of pesticide exposure commonly used in other organisms, 2) the impact of confounding biological and environmental factors on natural variations of biomarker responses, 3) the development of new biomarker approaches to assess long-term exposure to pesticides, and 4) the need of predicting adverse effects at individual and population levels from sub-individual biomarkers related to behaviour and reproductive disruption.

Need for Field Studies

Inhibition of B-type esterase activity has been traditionally the main biomarker of pesticide exposure. According to Aldridge's classification, B-esterases comprise hydrolases that are inhibited by organophosphorus (OP) and carbamate (CB) pesticides.[18] The most popular B-esterases in ecotoxicology are acetylcholinesterase (AChE, EC 3.1.1.7), butyrylcholinesterase (BChE, EC 3.1.1.8) and carboxylesterases (CbE, EC 3.1.1.1). AChE activity is a key enzyme in the normal functioning of cholinergic synapses at the nervous system and neuromuscular junction. It hydrolyzes efficiently the neurotransmitter acetylcholine, and it is considered a biomarker of pesticide exposure and toxic effect depending on the degree of AChE inhibition. BChE activity is particularly abundant in the blood of many vertebrate species and it hydrolyzes a wide variety of cholinesters.[19] This B-esterase has not a well defined physiological function and its inhibition by OP or CB pesticides does not lead to toxic effects. For this reason, BChE activity is considered a biomarker of pesticide exposure. CbE activity comprises multiple isozymes that participate in the detoxification of pesticides by two mechanisms: hydrolysis of the ester bond (CB, pyrethroids and some OP pesticides) and binding of the pesticide to the enzyme's active site (CB and OP pesticides). The inhibition of CbE activity by OP and CB pesticides takes place in the same way as for AChE or BChE activities.[20] In addition, the role of CbE in the detoxification of OP, CB and pyrethroid pesticides has led to consider this B-type esterase as a biomarker of susceptibility because levels of the enzyme activity and isozyme abundance appear to be related to pesticide tolerance.[21]

As with other organisms, earthworm AChE inhibition has been one of the most studied biomarkers of pesticide exposure in the past.[22–24] Some laboratory studies have shown that the measurement of AChE inhibition in earthworms is a sensitive biomarker to assess exposure to OPs and CBs (Table 1). Similarly, CbE activity has been explored in earthworms exposed to pesticides. As with mammals,[25] multiple CbE isozymes have been found in *L. terrestris* and a tissue-specific pattern of CbE isoforms was revealed by native polyacrylamide electrophoresis and subsequent staining for esterase activity using α-naphthyl acetate.[26] Up to seven CbE isozymes were distinguished in the reproductive tissue and body wall muscle, whereas only three isoforms were detected in intestine. Oien and Stenersen also found four and three esterase isozymes in *Eisenia unicolor* and *E. fetida*, respectively. The authors identified these isozymes as CbEs because they were fully inhibited by paraoxon and showed high activity toward α- and β-napththyl acetate.[27] However, no data are still available on the individual responses of these multiple CbE isozymes as well as changes in their abundance and tissue distribution when earthworms are exposed to pesticides. Despite these enzymological studies, the use of CbE inhibition to assess the anti-ChE pesticide impact on earthworms has not been as popular as ChE inhibition. Further investigations are still necessary to test some qualities of a suitable biomarker including sensitivity of CbE activity to inhibitory effects from anti-ChE pesticides, recovery rate of inhibited CbE activity, chemical reactivation with oximes (a specific diagnosis of OP toxicity), or relationship between CbE response and adverse effects at individual level.

Earthworm biomarker investigations have been mainly performed in the laboratory, and there is a need to test reproducibility of biomarker responses in the field. A few studies have examined the response of earthworm ChE activity against agricultural OP applications. Booth *et al.* found that natural populations of juvenile *A. caliginosa* as well as specimens caged within areas sprayed with the insecticides Lorsban 40EC (chlorpyrifos), Basudin 600EW (diazinon) or Carbaryl 80W (carbaryl) did not show inhibition of ChE activity after 2, 7 and 28 days of pesticide exposure.[28] The authors attributed this lack of ChE response to a high interindividual variation of normal ChE activity making the detection of individuals with inhibited ChE difficult. Conversely, a microcosm experiment showed that Dursban (chlorpyrifos) sprayed at the recommended application rate inhibited ChE activity of adults *A. caliginosa* during the two weeks following OP spraying.[29] A high dose-dependent relationship between chlorpyrifos concentrations in soil and ChE inhibition was found after 24 hr of spraying, but such a relationship was lost after 1 week of treatment, although ChE activity still remained depressed. A similar field study was carried out in a plum orchard sprayed with the same OP insecticide.[30] Inhibition of ChE activity of adults *A. caliginosa* caged in the chlorpyrifos-treated area was detected one to two weeks after spraying, with greater response evident at two weeks. These two microcosm

Table 1. Laboratory experiments on responses of earthworm cholinesterase activity to sublethal concentrations of pesticides

Species	Pesticide	Exposure level	Test conditions	Responses	Ref.
Eisenia andrei (Adults)	Carbaryl	4×10^{-3}, 0.48 and 48 mg a.i./kg (carbaryl). 4.29, 42.9, and 429 mg a.i./kg (Zoril 5). 1, 3 and 5 days	Natural soil. Moisture=24% Temp.=18±1°C. pH=8.0	– Dose-dependent inhibition of AChE activity, but no time-response relationship. – Maximal AChE inhibition (85.3%) at the highest pesticide concentration. – AChE inhibition at the highest dose of Zoril 5 (45–63%) after 24 hr, but decreased at 3 and 5 days after CB exposure.	85
Eisenia fetida (Adults)	Monocrotophos (Azofrin)	100, 150, 200 and 250 mg/kg 1, 7, and 14 days	OECD artificial soil. Moisture=35% Temp.=20±2°C. pH=6.0±0.5	– Dose-dependent inhibition of AChE activity. – Maximum AChE inhibition in the group exposed to 250 mg/kg after 14 days (90%). – Relationship between AChE inhibition and morphological damage.	86
E. fetida (Adults)	Chlorpyrifos	0.047 and 0.037 μg a.i./cm^2 12, 24, 36, and 48 hr	Paper contact test. (OECD, method 207)	– Inhibition of AChE activity (>60%). – Correlation of AChE inhibition with morphological damage.	87
Drawida willsi (Juveniles)	Butachlor (Bu), malathion (Ma) and carbofuran (Ca)	1.1 and 2.2 mg a.i./kg (Bu and Ca). 2.2 and 4.4 mg a.i./kg (Ma). 1–105 days	Natural soil. Moisture=20%. Temp.=25±2°C. pH=6.8	– Butachlor did not change AChE activity. – Inhibition of AChE activity during 30 days (malathion) and 60 days (carbofuran). – Maximum AChE inhibition after 9 days of malathion (41–46%) and after 12 days of carbofuran (54–63%) exposures.	88
Aporrectodea caliginosa (Juveniles)	Diazinon (Basudin 600 EW), Chlorpyrifos (Lorsban 40 EC)	12 and 60 mg a.i./kg (diazinon). 4 and 28 mg a.i./kg (chlorpyrifos). 28 days	Natural soil. Moisture=25% Temp.=20°C. pH=6.5–7.0	– 75 and 90% ChE inhibition at 12 and 60 mg/kg diazinon, respectively. – 35 and 70% ChE inhibition in individuals exposed to 4 and 28 mg/kg chlorpyrifos, respectively.	89
E. andrei (Adults)	Carbaryl	12, 25 and 50 mg a.i./kg 2, 7, and 14 days	OECD artificial soil. Moisture=35% Temp.=20±1°C. pH=6.0±0.5	– Inhibition (>60%) of AChE activity in all treatments and exposure times, but no clear dose (or time)-response relationship.	90
A. caliginosa (Juveniles)	Diazinon (Basudin 600 EW). Chlorpyrifos (Lorsban 40 EC).	60 mg a.i./kg diazinon. 28 mg a.i./kf chlorpyrifos. 1, 2, 4, 7, and 14 days	OECD artificial soil. Moisture=20–25 % Temp.=20°C. pH=6.5–7.0	– Inhibition of ChE activity in earthworms exposed to both pesticides after 24 hr (86% for chlorpyrifos and 75% for diazinon). – Level of ChE inhibition remained below 85% during the 2 weeks of exposure irrespective of the OP type.	28
E. fetida (Adults)	Fenitrothion	10 μg a.i./cm^2 24 hr	Paper contact test. (OECD, method 207)	– 89% of ChE inhibition.	91

studies have demonstrated the potential use of earthworm ChE inhibition to detect exposure to chlorpyrifos as long as one week after OP application. The application rate of chlorpyrifos and the earthworm age, among other possible factors, could account for the discrepancy in the ChE response of *A. caliginosa* found between the study by Booth *et al.*[28] and those by Reinecke and Reinecke.[29,30] Nevertheless, further field studies involving earthworm biomarkers are still required to know the potential applications of these biological measurements, in particular ChE inhibition, to assess pesticide exposure.

Persistence of biomarker response in time is a desirable feature of a suitable biomarker for field monitoring purposes. Many biomarkers show a transient temporal response to pollutants, but ChEs usually form a very stable complex when inhibited by OP pesticides and synthesis of new enzyme is the primary mechanism for returning esterase activity to its normal level.[12] Phosphorylated AChE of aquatic organisms generally recovers its normal activity within 1–2 weeks, although blood BChE activity in birds requires a few days to fully recover after acute OP exposure.[31] Earthworms are among the organisms that show the slowest recovery rates, in terms of several months, of phosphorylated ChEs.[5,32] This extremely slow recovery makes ChE inhibition an excellent biomarker of pesticide contamination. However, one major limitation of ChE activity comparisons for identification of pesticide-exposed individuals is the normally high variation of esterase activity between individuals. The obligation to use a well represented, non-exposed control group usually requires a great sampling effort. Some field studies with birds and reptiles have used the chemical reactivation of phosphorylated ChE activity with oximes as a complementary and specific diagnostic index of OP toxicity.[33–35] Generally, the sample tested for ChE inhibition by OPs is incubated with oximes such as pyridine-2-aldoxime methochloride (2-PAM). An increase of ChE activity in the 2-PAM-treated sample compared to the corresponding control (without the oxime) is attributed to the ability of this nucleophilic compound to remove the OP bound to the active site of the esterase. Similarly, oximes are able to reactivate OP-inhibited CbE activity. Maxwell *et al.* found that the oxime diacetylmonoxime reactivated more efficiently the phosphorylated plasma CbE activity of rat than other types of oximes such as 2-PAM and *N*,*N*′-trimethylenebis(pyridine-4-aldoxime).[36] Because this methodology enables each sample to act as its own control, it becomes a complementary approach when earthworm population density is low at the site of interest, and it solves the problem of high interindividual variation of ChE activity. However, the use of ChE-reactivating agents requires a previous optimization of the assay conditions (*e.g.*, 2-PAM concentration and time of incubation) to maximize the oxime-induced reactivation of phosphorylated ChE. In addition, the ability of 2-PAM to reverse phosphorylated ChE activity is highly dependent on the time elapsed since OP exposure.[33] Loss of one alkyl group from the OP-ChE complex occurs when the ChE has undergone prolonged phosphorylation; a process known as aging. At this stage, the oxime is not able to reactivate the ChE activity. The potential use of 2-PAM as a complementary methodology to assess exposure of earthworms to OP pesticides has been recently investigated in *E. fetida* and *L. terrestris*.[37] Phosphorylated earthworm ChE activity was reactivated with 5×10^{-4} M of 2-PAM, and the efficiency of the oxime to induce ChE reactivation was dependent on the OP type involved in the enzyme inhibition. Moreover, this study showed that chlorpyrifos-inhibited ChE activity displayed a slow aging rate and consequently 2-PAM-induced reactivation was possible for as long as one week after chlorpyrifos inhibition. Nevertheless, this attractive methodology for assessing OP exposure in earthworms still needs to be validated under field conditions.

Impact of Confounding Factors on Biomarker Responses

The influence of biological (life stage, sexual development, starvation, *etc.*) and environmental variables (temperature, moisture, pH, *etc.*) should be investigated when biomarker responses are used for field monitoring of pesticide contamination. This aspect of biomarker research has been extensively studied in vertebrates such as birds and fish,[10,31] and with certain biomarkers such as cytochrome P450-dependent monooxygenases (CYP) induction.[38] To date, only a few studies have examined the impact of confounding factors on earthworm biomarkers. Booth *et al.* found that the earthworm age (1, 2 and 3 months old) and soil type (loamy, sandy and clay soils) did not affect ChE activity, whereas soil temperature had a marked effect.[28] The ChE activity increased in earthworms acclimatized at 5°C and 20°C compared to those kept at 10°C. The seasonal effect on ChE activity was examined in *A. caliginosa*, *Aporrectodea nocturna*, *Allolobophora chlorotica* and *L. terrestris*, and a significant decrease was found only in *A. nocturna* sampled in autumn compared to spring.[39] The CbE activity of *A. caliginosa* also showed a significant variation between spring and autumn, whereas such seasonal variation was not observed in *E. fetida*.[40] The pattern of AChE isoforms changed according to the estivating (*i.e.*, inactivity stage occurring during prolonged periods of drought or heat) condition of the earthworm *A. caliginosa*. The estivating earthworms had four different AChE isoforms in both body wall and seminal vesicles compared to non-estivating individuals.[41] It was suggested that this change in the number of AChE isoforms might be associated with a possible role of AChE in the activation of collagenase during the estivation period.

The occurrence of multiple ChE isozymes with different sensitivity to pesticides can be considered as a confounding factor when they co-exist in the same tissue used for assaying. This is a frequent observation with ChEs of aquatic invertebrates. For example, Bocquené *et al.* isolated two different

ChEs in several tissues of the oyster *Crassostrea gigas*.[42] Despite differences in molecular weight, kinetic parameters and cellular location, one of them (called A-type ChE by the authors) was sensitive to some OP and CB pesticides, whereas the other (B-type ChE) was resistant to these compounds. The authors even suggested that treatment of the sample with 1 mM of paraoxon would enable the distinguishment of both ChE activities. A similar approach has also been applied to some fish species. Due to the presence of both AChE and BChE activities in the body muscle of *Gasterosteus aculeatus* that are able to hydrolyze the prototype substrates for enzymatic assay, a previous treatment of the sample with tetraisopropyl pyrophosphoramide (iso-OMPA) is required in order to distinguish both ChE activities.[43] Tissue distribution and biochemical characterization are therefore a recommended preliminary phase before the use of esterase inhibition as a biomarker for pesticide contamination.

Enzymological characterization of earthworm ChEs follows the strategy commonly used for distinguishing mammalian ChE activity.[44] Mammalian AChE activity preferentially hydrolyses the substrate acetylthiocholine iodide (AcSCh), and to a lesser extent, butyrylthiocholine iodide (BuSCh). It is inhibited at high substrate concentrations or by the specific inhibitor 1,5-bis(4-allyldimethyl-4-ammonimphenyl)pentan-3-one dibromide (BW284c51). On the other hand, mammalian BChE activity hydrolyses BuSCh at a higher rate than AcSCh or propyonylthiocholine iodide (PrSCh), and it is selectively inhibited with iso-OMPA or ethopropazine. However invertebrate ChEs often do not follow these enzymological criteria.[42,45] In the past, *in vitro* studies characterized earthworm ChEs. Stenersen found at least two main ChE activities in *E. fetida*, which were termed E1 (an AChE activity with preference for PrSCh, inhibited by BW284c51 and insensitive to iso-OMPA) and E2 (a BChE activity inhibited by ethopropazine and by substrate excess).[24] Three AChE forms were isolated by chromatographic techniques from *A. caliginosa*, and they were suggested as monomeric, dimeric and tetrameric forms of AChE instead of different isozymes.[23] Five different AChE forms corresponding to polymerization of AChE monomers were also isolated by polyacrylamide gradient gel electrophoresis from *E. fetida*.[46] These studies and other more recent investigations showed that earthworm ChE activity should be referred as a true AChE because of its response to BW284c51 (inhibition) and iso-OMPA (no effect), and its preference for the substrate PrSCh.[39] However, a BChE activity showing some different enzymological properties than those of mammals can be also present in some earthworm species.[24,39]

Tolerance to Pesticides: A Biomarker-Based Approach to Assess Long-Term Effects

Most of the laboratory studies with biomarkers employ short-term (<1 month) exposure protocols and relatively high pollutant concentrations. This approach is valid for assessing acute exposure to contaminants. The most realistic situation in the environment, however, is that organisms are exposed to low concentrations of contaminants, and occasionally for long periods of time. Soil serves as a sink for many pollutants such as pesticides and therefore long-term exposure by soil biota is plausible. One of the chronic effects from sublethal long-term exposure to pesticides is the development of resistance; a phenomenon extensively studied in some species of insect pests. An over-expression of pesticide-metabolising enzymes such as CbE, phosphotriesterases (PTEs), gluthation *S*-transferase (GST) and CYP as well as a decreased sensitivity of the target site (*e.g.*, AChE), are the main molecular mechanisms for developing insecticide resistance.[21,47] CYP comprises a superfamily of hemoproteins involved in the oxidative metabolism of pesticides, among other endogenous and exogenous compounds. CYP also catalyzes the bioactivation of phosphorothioate- and phosphorodithioate-type OP pesticides to their highly toxic 'oxon' form.[48] Two CYP subfamilies are present in earthworms, *i.e.*, the polyaromatic hydrocarbon-inducible form (CYP1A) and the phenobarbital-inducible form (CYP2B),[49] but they show relatively low level of activity compared to mammals or fish.[50] Moreover, earthworm CYP activity did not exhibit induction by potential inducers of mammalian CYP activity (Table 2). The role of earthworm CYP activity as a biomarker of pesticide contamination is therefore uncertain, and it does not seem to play a significant role in pesticide detoxification and bioactivation. GST activity also plays an important role in the metabolism of xenobiotics. This multigene family of cytosolic enzymes catalyzes the conjugation of electrophilic metabolites with the tripeptide glutathione to yield a water-soluble conjugated metabolite. Unlike CYP activity, earthworms have a relatively high GST activity compared to mammals,[49] but its use as biomarker of pesticide exposure is also questionable. Earthworm GST activity is not induced by prototypical inducers of mammalian GST.[51] The same result has been obtained with earthworms exposed to OP pesticides and carbaryl. Conversely, organochlorine pesticides seem to induce earthworm GST activity (Table 2). The implications of earthworm PTEs and CbEs in pesticide metabolism and toxicity have not been studied in detail, although PTE activity has been well characterized in some earthworm species (Table 2). Some investigations have suggested that the abundance of CbE isozymes and relatively high levels of enzyme activity can contribute to the tolerance of the organism to toxic effects from anti-ChE pesticides.[19,52–54] High levels of PTE activity equally contribute to pesticide tolerance; these esterases are the main mode of OP detoxification in many organisms.[20,55]

Considering the role of PTEs, CbEs, GST and CYP activities in the metabolism of pesticides, it would be feasible to assume that species-specific differences in pesticide toxicity, or tolerance, might be due to differences in enzyme activity levels as well as the induction capacity. Although this assumption is true for some pest species, the question arises as to

Table 2. Baseline levels and response of earthworm pesticide-metabolizing enzymes to pollutants[a)]

Enzyme	Role in pesticide metabolism	Species	Xenobiotic and exposure level	Response and occurrence	Ref.
CYP1A CYP2B	Phase-I detoxification (oxidation, dealkylation, epoxidation, hydroxylation).	*Dendrobaena veneta*	3-MC (75 μg/g) and PB (75 μg/g). 72 hr after intracoelomic injection (3-MC) or 24 hr after multiple injections (PB).	No induction (total content of microsomal CYP).	92
		Lumbricus terrestris	Aldrin (5 μg/g). 48 hr after intracoelomic injection.	High aldrin epoxidase activity in the intestine.	93
	Bioactivation of OPs (oxidative desulfuration).	*Eisenia fetida*	Fluoranthene and benzo(a)pyrene (10 and 100 mg/kg dry wt). 28 days of exposure to contaminated soil.	No change in microsomal PROD and BROD activities. No microsomal EROD activity.	94
		Lumbricus rubellus	Pyrene (10–2,560 mg/kg dry wt). 42 days of exposure to contaminated soil.	No microsomal EROD activity.	95
		E. fetida	Benzo(a)pyrene and pyrene (10^{-6}–10^{-2} mg/ml). 48 hr of exposure to contaminated filter paper (OECD, method No. 207).	Significant increase of total content of microsomal CYP.	96
		Eisenia andrei	Carbaryl (12–50 mg/kg dry wt). 2, 7, and 14 days after exposure to contaminated soil.	Inhibition of microsomal MROD activity.	90
		Aporrectodea tuberculata	Metal (Cu and Zn) contaminated soils. 2, 7, and 14 days of exposure.	Dose- and time-dependent induction of microsomal EROD activity.	97
GST[b)]	Phase-II detoxification (conjugation).	*E. andrei*	3-MC (5×10^{-5} M), TSO (1×10^{-4} M), and TCPOBOP (3×10^{-5} M). 24 hr after multiple treatments.	No induction of GST activity. Presence of 4 GST isozymes. Mean basal GST activity=529 nmol/min/mg protein.	51
		Pheretima posthuma	Aldrin (1 mg/kg), endosulfan (1 mg/kg) and lindane (1 mg/kg). 4 weeks after exposure to contaminated soils.	GST induction (26–151% compared to controls) for all organochlorine pesticides during 2 weeks of exposure. Mean basal GST activity=11.3 μmol/min/mg protein.	98
		E. andrei	Carbaryl (12–50 mg/kg dry wt). 2, 7, and 14 days after exposure to contaminated soil.	No effect upon GST activity. Mean basal GST activity=381 nmol/min/mg protein.	90

Table 2. Continued.

Enzyme	Role in pesticide metabolism	Species	Xenobiotic and exposure level	Response and occurrence	Ref.
		Aporrectodea caliginosa	Diazinon=12 and 60 mg a.i./kg. Chlorpyrifos=4 and 28 mg a.i./kg. 28 days after exposure to contaminated soils.	Chlorpyrifos did not affect GST activity, whereas diazinon inhibited GST activity at both concentrations. Basal GST activity=49–101 nmol/min/mg protein.	89
		E. fetida	Fenitrothion=10 μg a.i./cm^2 24 hr after exposure to contaminated filter papel (OECD, method No. 207).	No effect on GST activity. Mean basal GST activity=227 nmol/min/mg protein.	91
PTEs	Hydrolysis of OP pesticides.	*E. andrei*	Cultured earthworms.	90% of PTE activity is present in the intestine.	99
		L. terrestris[c]	Cultured earthworms.	Presence of phosphomonoesterase (14.7 nmol/min/mg protein) and phosphodiesterase (320 pmol/min/mg protein) activities able to hydrolyze OP pesticides.	100
CbEs	Hydrolysis of pyrethroid and CB. pesticides.	*E. fetida*	Fenitrothion (10 μg a.i./cm^2). 24 hr after exposure to contaminated filter paper (OECD, method No. 207).	95% of CbE inhibition. Mean (±SD) basal CbE activity[d]=443.9±13.3 nmol/min/mg protein.	91
	Binding to CB and OP pesticides.	*E. fetida*, *A. caliginosa*	Natural populations.	Mean basal CbE activity[e] of *E. fetida*=6.2 nmol/min/mg protein. Mean basal CbE activity[e] of *A. caliginosa*=7.5 nmol/min/mg protein.	40

[a] Abbreviations are: CYP1A=polyaromatic hydrocarbon-inducible CYP form, CYP2B=phenobarbital-inducible CYP form, GST=glutathione *S*-transferase, PTEs=phosphotriesterases, CbEs=carboxylesterases, 3-MC=3-methylcholanthrene, PB=phenobarbital, TSO=*trans*-stilbene oxide, TCPOBOP=1,4-bis[2-(3,5-dichloropyridoxyl)]benzene, PROD=penthoxyresorufin *O*-deethylase, BROD=benzoxyresorufin *O*-deethylase, MROD=methoxyresorufin *O*-deethylase. [b] 1-Chloro-2,4-dinitrobenzene (CDNB) as substrate. [c] Activity is expressed as nmol *p*-nitrophenol formed/min/mg protein. [d] Phenylthioacetate as substrate. [e] *p*-Nitrophenyl acetate as substrate.

what extent these biochemical markers are really workable in non-target organisms such as earthworms. Carboxylesterases and PTEs still offer an exciting field of investigation with earthworms, not only because of the need for information (Table 2), but also because they could result in suitable biomarkers of long-term exposure that yield significant information on population effects in terms of tolerance or resistance to pesticides. For example, gene amplification and changes at the level of transcriptional regulation usually explain the elevated production of CbE in insecticide-resistant organisms.[47] Whether this enhanced CbE-mediated detoxification of pesticides also occurs in natural populations of earthworms chronically exposed to agrochemicals remains to be examined. This biomarker-based approach to assess long-term exposure to pesticides in earthworms may be a suitable strategy to assess the adverse effects from low concentrations of pollutants and long exposure times; one of the current challenges in ecotoxicology.[56]

Perspectives in Earthworm Biomarkers

Molecular or biochemical biomarkers are usually either the target for acute toxicity (*e.g.*, brain AChE) or the enzymatic systems involved in xenobiotic detoxification (CYP, CbEs, GST, metallothioneins, *etc.*). Biomarkers belonging to the latter group are considered to be biomarkers of exposure because they do not give a reliable prediction of toxic effects. Induction or inhibition of xenobiotic-metabolizing enzymes is a clear signal of pollutant exposure, but it does not mean that the individual health is seriously at risk. There is still a need for establishing a direct link between sub-individual biomarkers and adverse effects at individual or population level. Biomarkers directly related to behavioural changes or reproductive success could be a chance for such a relationship. Based on biomarker studies involving other organisms and data from other fields of research, we have proposed a set of molecular and biochemical measurements that could be potential biomarkers of pesticide toxicity at behavioural and reproductive levels.

Biomarkers of behavioural disruption. Organisms severely affected by toxicants can display an altered pattern of their normal behaviour (locomotion, feeding, escape from predators, sexual activity, *etc.*). Some investigations show that earthworms exposed to pesticides display marked changes in burrowing,[57] surface migration,[58] feeding activity,[59] and avoidance ability.[60,61] These pollutant-induced behaviour changes in earthworms can lead to indirect effects on population dynamics (biomass, density, age-class structure), soil properties (nutrient cycles, aeration or drainage) or even plant growth (shoot and root biomass).

Avoidance, burrowing and surface migration require locomotor activity. Earthworm locomotion is often called fictive locomotion because the rhythmic motor activity, generated in the central nervous system, takes place without any locomotor organ or structure.[62] The earthworm fictive locomotion is induced by the neurotransmitter octopamine (OA).[62–64] This biogenic monoamine, generally found at high concentrations in the tissues of many invertebrates, is involved in a wide range of invertebrate behaviours.[65] Although the earthworm fictive locomotion seems to be controlled by OA, no studies have explored whether pesticides are able to change levels of this monoamine, affecting probably the earthworm locomotor activity. However, OP pesticides are able to alter the levels of the monoamines dopamine, serotonin or adrenaline in rats,[10] and decrease the brain concentrations of tyrosine, the amino acid precursor for synthesizing OA.[67] Although the effects of OP pesticides on monoamine levels have been studied in mammals, these investigations serve as a stimulus for investigating similar effects of OPs upon earthworm OA levels correlated with locomotor impairment. If such a relationship is established, then OA could be considered a biomarker of behaviour disruption.

Inhibition of AChE activity by OP and CB pesticides is another biomarker directly implicated in behaviour perturbation. This relationship has been extensively investigated in aquatic invertebrates,[10,19] fish,[67] and mammals.[68] Earthworm body wall muscles present vertebrate-like cholinergic neuromuscular junctions,[69] which contains the enzyme AChE for regulating the synaptic transmission. Despite an intuitive connection between inhibition of muscle AChE activity by anti-ChE pesticides and perturbation in locomotor activity, there are few studies that show such a relationship with earthworms.[70]

Pesticide-induced changes in earthworm behaviour and biomarker response (*e.g.*, changes in OA levels and inhibition of AChE activity) can be examined by the experimental design illustrated in the Fig. 1. In general, earthworms avoid contaminated soils and this behaviour has been used to develop a standardized screening test for assessing potential toxicity of contaminated soils.[71] Different designs have been used for the avoidance behaviour response (ABR) test,[72,73] although the most popular and simplest experimental setup consists of two equal compartments separated by a removable split (Fig. 1A). Reference and contaminated (or pollutant-spiked) soils are placed in each compartment, and once the separator is removed the earthworms are released just in the space initially occupied by the separator and then individuals are counted in each soil 48 hr later. However, this standardized protocol for the ABR test minimizes the contact of the earthworms with the contaminated soil unless pollutant concentrations are too low to cause a repellence reaction in the organisms (Fig. 1A). A study with fish demonstrated that pre-exposure to contaminants reduced the ability of fish to avoid the polluted environment.[74] After pesticide applications, evading ability of anecic or endogeic earthworms from pesticide-contaminated soils implies vertical and horizontal movements. In a speculative context, migration over the soil surface can lead to a higher risk of pesticide exposure and the capacity to avoid the contaminated soil could become diminished. The standardized ABR test does not consider this risk. The meaning of this be-

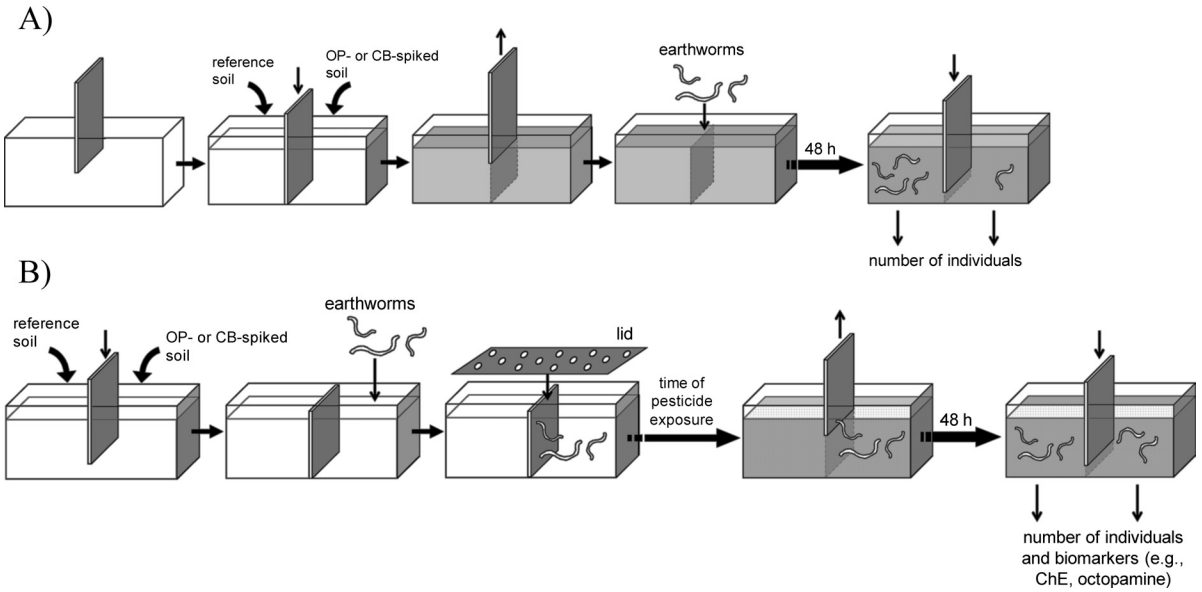

Fig. 1. A) Standardized protocol for behaviour avoidance response test with earthworms (adapted from Loureiro *et al.*).[73] B) Tentative experimental procedure for assessing impact of pesticides upon avoidance ability of earthworms. This alternative test enables to link sub-individual biomarkers related to locomotor activity (*e.g.*, cholinesterase inhibition or octopamine levels) with avoidance capacity.

havioural test can be modified if earthworms are previously released in the compartment containing the contaminated soil and, after a fixed period of time, the separator is removed to enable earthworms move toward the clean soil (Fig. 1B). This approach would allow assessment of the impact of pesticides on the ability of earthworms to avoid polluted soils, which would imply a high sensitivity of chemoreceptors and efficiency in locomotor activity. The proposed experimental design in Fig. 1B could provide solid evidences for a link between inhibition of ChE activity, or change in OA levels, and locomotor impairment. With this slight modification in the experimental procedure of the ABR test, the assay would become a toxicity behaviour test and not merely a screening test.

Biomarkers of reproductive disruption. The isolation of the neuropeptide annetocin from *E. fetida* has initiated a new line of promising research in the field of earthworm biomarkers.[75] This oxytocin-related peptide seems to stimulate earthworm behaviours associated to cocoon formation and egg laying.[76] Gene expression of annetocin has been investigated in *E. fetida* exposed to soils contaminated with heavy metals.[77] Earthworms exposed to Pb/Zn-contaminated soils showed a 20-fold reduction of annetocin gene expression compared to controls, besides a strong reduction of cocoon production. This study encourages using the annetocin as a molecular biomarker of reproductive disruption. The response of annetocin expression levels have not yet been tested with pollutants other than heavy metals. Another potential biochemical biomarker that could be related to reproductive fitness is CbE in the reproductive tissues. Carboxylesterase over-expression is a common feature in the male reproductive system of organisms as dissimilar as rodents, bivalve molluscs and insects.[78] This CbE over-expression seems to have an important role in the spermatogenesis and male reproductive health. An interesting review by Mikhailov and Torrado suggests that CbE activity levels in the male reproductive system could be a determinant in the local protective mechanism for sperm differentiation and maturation against pesticides such as OP, CB and pyrethroids.[78] Generally, high CbE activity levels in vertebrates are associated with tolerance to pesticides[79]; therefore, it would be not unwise to assume that toxic effects on the reproductive system would be correlated to CbE activity levels in this tissue. Many studies show that pesticides currently used in modern agriculture (*e.g.*, anti-ChE and pyrethroid pesticides) are able to cause reproductive toxicity in earthworms. Severe damage in the spermatid and spermatozoa morphology and development have been described in earthworms exposed to benomyl,[80] dieldrin,[81] and imidacloprid.[82] In addition, CbE activity is well represented in the reproductive tissue of these organisms compared to other tissues such as the intestinal tract.[26] The role of CbE activity in the earthworm reproductive system needs to be explored in order to examine whether levels of these esterases or affinity to anti-ChE pesticides really respond to a protective mechanism of reproductive system against these pesticides.

Conclusions

Earthworm biomarkers of heavy metal exposure have experienced a significant progress in the last decade.[16,83,84] Earthworms are common organisms in agroecosystems with direct beneficial effects on plant growth and soil functioning. Paradoxically, very few studies have assessed the impact of agricultural pesticide applications upon earthworm populations

through the use of biomarkers. On the other hand, several reviews have stressed the need for a greater understanding of earthworm biomarkers of organic contaminants of current concern such as anticholinesterase and pyrethroid pesticides.[17,83] Although some traditional biomarkers of pesticide exposure such as AChE or CbE inhibition have been explored in earthworms, there are a large number of experimental questions that still need to be addressed. For example, it is necessary to know the impact of abiotic and biotic factors on biomarker responses to avoid false interpretations when used in natural earthworms populations exposed to pesticides. Environmental factors such as temperature, moisture or pH can alter the lethality of pesticides. In many cases, such an interaction is the result of changes in the pesticide degradation rate leading to a less exposure level, but in others, the mechanisms responsible for alterations in pesticide toxicity can be related to biotic factors (*e.g.*, changes in bioactivation and detoxification processes and pesticide uptake). Laboratory experiments are therefore needed to examine to what extent abiotic variables influence the normal fluctuation of earthworm biomarkers, particularly those biomarkers related to pesticide metabolism (*e.g.*, CbE or GST activities). These mechanistic studies would provide valuable indication on synergistic interactions between environmental stressors and pesticide toxicity.

Organophosphates are one of the most common groups of agrochemicals currently applied in agriculture. These pesticides show a relatively short persistence in the environment and within the organism, which makes difficult their detection in both abiotic and biotic samples by chemical analysis. Additional methods of OP exposure assessment are therefore required for field monitoring. In this way, the oxime-induced reactivation of phosphorylated ChE activity results a complementary and specific methodology of exposure to OP pesticides. For example, field studies with birds and reptiles have shown that this diagnostic index of OP toxicity enabled to identify exposed individuals who ChE activity levels were not significantly different from those of non-exposed individuals.[33–35] Reactivation of earthworm phosphorylated ChE activity with 2-PAM has revealed as a promising tool for field monitoring of pesticide.[37] However, this attractive methodology requires field validation to be accepted as a suitable indicator of OP exposure, and furthermore, other oximes (*e.g.*, obidoxime, diacetylmonoxime) and esterases (*e.g.*, CbE) should be tested.

Most biomarkers currently used in field monitoring provide an indication of pollutant exposure only. Thus, the use of a suite of biomarkers covering from molecular to whole-organism endpoints is a desirable strategy for assessing the impact of pesticide on individual health. We have presented some clues to apply this multibiomarker approach in earthworms. For example, the measurement of AChE inhibition and OA levels (molecular/biochemical biomarkers) together to the avoidance behaviour response (behavioural biomarker) could be a suitable multibiomarker approach to assess the impact of pesticides upon earthworms. Similarly, the measurement of CbE activity levels in the reproductive tissues together to cellular biomarkers such as sperm number and morphology may be an attractive multibiomarker approach to assess pesticides implicated as possible endocrine disruptors.

Despite their current use in field monitoring of pesticide contamination, biomarkers could occupy an important place in laboratory toxicity testing. Predictions about toxicity of new pesticides before can be authorized for use or the ecological risk assessment of pesticide-contaminated soils require toxicity tests with earthworms (*e.g.*, European Council Directive 91/414/EEC).[101] The main toxicity endpoints in earthworm toxicity bioassays are survival, growth change and reproduction. These toxicity endpoints can be insufficient when pesticide concentrations in soil are relatively low and chronic effects cannot be predicted from the standardized short term laboratory tests. The inclusion of the biomarker approach in these laboratory tests would increase the meaning of the test outcomes in terms of pesticide bioavailability and sublethal effects. In addition, predictions about long-term population effects and indirect effects of pesticides can be formulated whether biomarkers of behaviour and reproductive disruption are workable in the short-term toxicity bioassays.

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