Subject Area 7.2: Evaluation of the impacts and interactions of chemicals in the laboratory or real environments, and their fate in biota, including animals and humans

Research Article

Effect of Herbicides on Glutathione S-transferases in the Earthworm, *Eisenia fetida**

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Abstract

Aim and Background. Earthworms have been studied as a readily available, easily maintainable and cheap test species for assessing chemical pollution, and may be an alternative to in vivo rodent bioassays. The current investigation aims to characterize detoxification enzymes in *Eisenia fetida* and stress response against two herbicides with different modes of action, namely, fenoxaprop and metolachlor.

Methods. Herbicides were applied to soil containing earthworms. Animals were then collected, sacrificed and shock-frozen. Extracted protein was analyzed for glutathione S-transferase (GST) activity using CDNB (1-chloro-2,4-dinitrobenzene), DCNB (1,2-dichloro-4-nitrobenzene), pNBC (p-nitrobenzylchloride), PNOBC (p-nitrobenz-o-ylchloride) and selected herbicides. GST isoenzymes were partially purified by affinity chromatography and molecular weights were estimated by SDS-PAGE.

Results. In *E. fetida* protein extracts, GST activity towards model compounds ranked as CDNB>DCNB>PNOBC>pNBC. Fluorodifen was not conjugated at all, but fenoxaprop and metolachlor were conjugated at low rates. Furthermore, the GST isoenzyme pattern changed during the incubation with herbicides, either due to stress or as a defense reaction. After incubation with monochlorobimane, a strong fluorescence of the intestinal tract and the intersegments was observed, indicating organ-specific GST induction.

Discussion. According to the author's knowledge, here, for the first time, evidence is presented that *E. fetida* GST are also capable of conjugating a wider range of xenobiotic substrates. Different forms of GST were observed and changes in GST isoforms due to the herbicide treatment were also noticed. GST conjugation rates varied between different herbicides used in this experiment. It might be assumed that herbicides may well be detoxified by earthworms, to a certain extent, but that they are also potent stress factors influencing the detoxification system of the animal. High doses or long exposure might lead to deleterious effects on earthworms and limit their survival rate. The use of the animals as bioindicators for herbicides and herbicide residues seems very promising, but is surely influenced by the lack of detoxification for some compounds.

Conclusions. Conjugation of several xenobiotics with model substances and herbicides is proven in the earthworm *E. fetida*. However, *E. fetida* has only limited capabilities of detoxifying herbicidal compounds. Different isoforms of GST were involved and altered in their activity after treatment.

Recommendations and Perspectives. The accumulation of GS-conjugates and their determination via fluorescence microscopy is a quick and secure, additional marker for exposure that should be further developed to complement existing biotests. The described methods and endpoints might help to understand the complex reaction of earthworms towards herbicides and lead to an adapted test methodology.

Keywords: Affinity chromatography; earthworm; *Eisenia fetida*; fenoxaprop; glutathione S-transferase isoforms; herbicides; metolachlor; monochlorobimane

Introduction

All living organisms contain enzymes and biomolecules with antioxidant capabilities to protect them against the adverse effects of xenobiotics. Glutathione and glutathione-s-transferase (GST) play a major role in cellular defense mechanisms. Glutathione S-transferases have been intensively studied for their involvement in herbicide detoxification and several GSTs are known to be responsible for herbicide tolerance in crops and resistance in weeds (Cole et al. 1994).

Several widely used herbicides, among them alachlor, metolachlor, propachlor or fenoxaprop are detoxified in biological systems by the formation of glutathione acetanilide conjugates. This conjugation is mediated by GST (Farenhorst and Bowman 2000). GSTs and glutathione interact perfectly in protecting cells from electrophilic xenobiotics and endogenous toxic compounds. They are also involved in hormone metabolism and intercellular signal cascades. The enzyme class shows a high flexibility and represents the starting point of well-developed metabolic pathways in the degradation and excretion of xenobiotic conjugates (Schröder 2001). The cytosolic as well as the microsomal GSTs of most species are dimeric enzymes consisting of subunits of the same or similar size between MW 23 and 30 kDa. The determination of subunit size is in most cases deduced from SDS-PAGE separation and migration (Schröder 2001).

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GSTs and glutathione seem to have co-evolved jointly as they are found ubiquitously in animals and plants. However, so far, little is known about the GST classes in earthworms.

Earthworms are universally accepted as one of the most suitable and representative soil animals to be used for assessing chemical pollution in soils, for evaluating environmental hazards and to evaluate the use or abuse of field applications of pesticides (Viswanathan 1997, Leland et al. 2001, Booth et al. 1998, Booth and O'Halloran 2001, Booth et al. 2001, Hund-Rinke and Wiechering 2001, Hund-Rinke et al. 2003). They have been studied as a readily available, easily maintainable and cheap test species to develop toxicity systems as an alternative to in vivo rodent bioassays (Furst 2002, Römbke et al. 2005).

GST activity has been demonstrated in six species of earthworms of the family Lumbricidae (Stenersen et al. 1979, Stenersen and Oien 1981, Stenersen 1984), although, in most cases, only the standard substrate, CDNB, a chlorinated benzene, has been utilized. The use of this substrate is under some debate, as it has been demonstrated that it would yield some information on general availability of glutathione S-transferases, but that differences in the detoxification capacity under the influence of stressors would probably not be detectable.

Eisenia fetida is an earthworm of high abundance in compost and the upper straw layer of soils. It has been used as a bio-monitor for heavy metals and insecticide residues in soils (OECD 1984). Very recently, earthworm ecotoxicology and potential spin-off for the use of earthworm data in eco-(toxico)logical study risk assessment gained much interest (Van Gestel and Weeks 2004). Their capability to respond to chemical stimuli (Schaefer 2003) has led to their use in avoidance tests, with very promising results (Hund-Rinke et al. 2005). Earthworms have also been exposed to soils spiked with the herbicide precursor, chloroacetamide, which was lethal to the animals (Hund-Rinke et al. 2003), with butachlor, which caused severe histological changes (Muthukaruppan et al. 2005), and with acetochlor (Xiao et al. 2006). Although used in these test systems, our current knowledge about *E. fetida* biochemistry and stress response is still restricted. To fill this gap, the current investigation aims at the characterization of GST isoforms in *E. fetida* and at the changes due to stress responses against 2 different modes of herbicide action, namely of fenoxaprop (FP) and metolachlor (MC).

1 Materials and Methods

Chemicals. All chemicals were the highest analytically available, herbicides fenoxaprop (FP) and metolachlor (MC) were Pestanal standards.

Earthworms. *Eisenia fetida* were obtained from a local grower near Munich. They were kept in compost soil mix under controlled greenhouse conditions (12 hrs dark: light conditions, relative humidity 60% and controlled temperature 25°C) in large 10 L flower pots.

Herbicide application. The herbicides fenoxaprop ((R)-2-[4-(6-chloro-2-benzoxazol-2-yloxy)phenoxy]propionate) and metolachlor ((S)-2-chloro-N-(2-ethyl-6-methyl-phenyl)-N-(2-

methoxy-1-methyl-ethyl)-acetamide) were applied to the earthworms in standard glass Petri dishes (80 x 15 mm) at concentrations of 2,700 mg FP/kg and 3,200 mg MC/kg body weight for 48 hrs. Concentrations applied were in the range of earlier experiments (e.g. Farenhorst et al. 2003). Each incubation was performed at least in triplicate with 30 worms (total fresh weight approx. 5 g) in each experiment. At the end of the incubations, after 24 hrs (10 worms) and after 48 hrs (20 worms), animals were collected, sacrificed and shock-frozen in liquid nitrogen.

Microscopy of fluorescent conjugates. In a separate experiment, and in order to evaluate the use of earthworms as bioindicators, the earthworms were exposed to a soil pretreated with fenoxaprop (100 mg/kg soil corresponding to 10fold recommended dose of 500 g ai/ha) in large glass beakers. After 14 days, the worms were collected, sacrificed and treated with monochlorobimane, a fluorescent probe for glutathione. The development of fluorescence was monitored in a fluorescence microscope (Leitz Axiovert) and recorded with a CCD camera.

Enzyme Purification. Frozen earthworms (-80°C) were ground to a fine powder in liquid nitrogen with mortar and pestle and added to ten volumes (v/w) of potassium phosphate buffer (100 mM, pH 7.8), containing 10 mM dithioerythritol, 5 mM EDTA, 1% Nonidet P40 and 10 mg·ml⁻¹ soluble polyvinylpyrrolidone K30. The slurry was incubated for 10 min, filtered through Miracloth (Calbiochem) and centrifuged at 30,000 × g for 30 min. Solid ammonium sulfate was added to the supernatant to 40% saturation. After stirring for 30 min, the precipitated proteins were collected by centrifugation at 30,000 × g for 25 min. The supernatant was decanted and adjusted to 80% ammonium sulfate saturation, stirred for 30 min and centrifuged as described previously. The resulting pellet was resuspended in 2.5 ml 20 mM Tris-HCl (pH 7.8; buffer A), desalted by gel filtration (PD 10 columns, Pharmacia) and then eluted with 3.5 ml of the same buffer.

Enzyme Assays and Protein Determination. All fractions collected from the chromatography step were analyzed for GST activity, determined spectrophotometrically using CDNB (1-chloro-2,4-dinitrobenzene), DCNB (1,2-dichloro-4-nitrobenzene), pNBC (p-nitrobenzylchloride) and PNOBC (p-nitrobenz-o-ylchloride) as model substrates according to Habig and coworkers (1974). For the determination of the GST-dependent conjugation of these model compounds, aliquots of the enzyme extract were incubated with 0.1 M Tris HCl buffer at pH 7.8, 1 mM GSH with 1 mM 1-chloro-2,4-dinitrobenzene (CDNB, $\epsilon_{340\text{ nm}} = 9.6\text{ mM}^{-1}\text{cm}^{-1}$) or 1 mM 1,2-dichloro-4-nitrobenzene (DCNB, $\epsilon_{345\text{ nm}} = 8.5\text{ mM}^{-1}\text{cm}^{-1}$), furthermore with p-nitrobenzylchloride (p-NBC, $\epsilon_{310\text{ nm}} = 1.8\text{ mM}^{-1}\text{cm}^{-1}$) and p-nitrobenz-o-ylchloride (p-NBOC, $\epsilon_{310\text{ nm}} = 1.9\text{ mM}^{-1}\text{cm}^{-1}$), resulting in a total assay volume of 0.6 ml. Controls lacking enzyme extracts or GSH were measured. Enzyme activity is expressed in units of kat (katal). 1 kat represents the enzymatic formation of 1 mol of end product per sec in the enzyme extracts. High-performance liquid chromatography (HPLC) of herbicide metabolites was performed with a Varian Model 230 HPLC System equipped with a Gynkotek Spectrometer SP-4 UV monitor at 280 nm.

For HPLC, a RP-C₁₈ Hypersil-ODS-column NC-04, 5.0 µm, 250 x 4.0 mm, radial cartridge (Bischoff) was used with acetonitrile/water with 0.1% trifluoroacetic acid solvent system. Peaks were eluted at a flow rate of 1 ml/min in a gradient from 60% acetonitrile for 5 min followed by a 15 min increase to 100% acetonitrile (Schröder et al. 1990). Protein contents were determined by the method of Bradford (1976), using bovine serum albumin as standard.

Affinity chromatography. GST isoenzymes were partially purified by affinity chromatography on epoxy-activated agarose with S-bound GSH as a ligand (Sigma-Aldrich). Here, care was taken to allow for equilibration of the enzyme fraction with the ligands of the chromatographic gel prior to elution. The elution of GST from the affinity chromatography matrix followed the pattern previously described (Schröder and Berkau 1993, Schröder et al. 2002). In short, combined enzyme fractions were desalted by gel filtration, centrifuged as described above and loaded onto GSH agarose columns (2.5 ml bed volume) pre-equilibrated with Tris-HCl (25 mM, pH 7.0), 1 mM dithioerythritol (buffer A). For the elution of GSTs, Tris-HCl buffer (25 mM, pH 7.0) was prepared with 20 mM GSH and 1 mM NaCl (buffer B). After sample application, the column was washed with five volumes of buffer A, to elute the non-bound protein, and bound GST was eluted by a step gradient of buffer B from 0 to 20 mM GSH. In order to further concentrate the protein in fractions (0.5 ml) containing GST activity, buffer B was replaced with water by gel filtration on Sephadex G25, and samples were lyophilized overnight.

Gel Electrophoresis. Molecular masses and purity of GST isoenzymes were estimated by SDS-PAGE using 8 to 25% gradient gels (Phast System, Pharmacia LKB). A low molecular weight marker kit (Sigma-Aldrich) was used as a standard for determination of GST subunit molecular weights. Lyophilized proteins were resuspended in small volumes of water (20 to 100 µl), diluted 1:1 with loading buffer, and proteins were denatured by boiling for 7 min. Resolved protein bands were silver stained according to Blum et al. (1987). Apparent molecular mass was determined by interpolating linear plots of log MW of markers versus R_f values.

Statistical treatment of data. Results were analyzed using SPSS and SigmaStat programs. Significance was tested by analysis of variance. Probability levels for statistical significance were $P < 0.05$. All results are presented as means $\pm$ SD.

2 Results

GST activities in the earthworm. The incubation of earthworms with selected herbicides (fenoxaprop, metolachlor) led to a strong avoidance reaction, where approximately 50%

Table 1: GST activities for several substrates in protein extracts of *E. fetida* after ammonium sulfate precipitation. Measurements were performed in triplicate and given $\pm$ SD

Substrate	Volume activity (µkat/ml)	Specific activity (µkat mg prot)
CDNB	11.28 $\pm$ 0.26	2.8 $\pm$ 0.16
DCNB	0.177 $\pm$ 0.009	0.045 $\pm$ 0.004
PNBC	0.0154 $\pm$ 0.0014	0.0039 $\pm$ 0.0004
PNBOC	0.408 $\pm$ 0.08	0.103 $\pm$ 0.022
Fluorodifen	0.0 ^a	0.0 ^a
Fenoxaprop	0.65 $\times 10^{-6}$	0.164 $\times 10^{-6}$
Metolachlor	350.0 $\times 10^{-6}$	88.6 $\times 10^{-6}$

^a no enzyme activity above the non enzymatic blank

of the animals tried to escape from the incubation vessels within 48 hrs.

In *Eisenia fetida*, protein extracts, highest GST activity was recorded for the model substrate CDBN, whereas the closely related model compound DCNB was conjugated 100 folds slower. PNBC, a nitrophenyl chloride known to be conjugated by few GST isoforms, was converted to glutathione conjugate at a rate of 0.0154 µkat/ml, whereas the chemically similar PNBOC was conjugated at a rate of 0.408 µkat/ml in the same extract (Table 1).

Three herbicides from different chemical families and with different mode of action were tested for conjugation with *E. fetida* extracts. Whereas fluorodifen was not conjugated at all in our experiments, the post-emergence herbicide fenoxaprop was conjugated at low rates. In comparison, the pre-emergence (soil application) acetanilide metolachlor was converted to a glutathione conjugate at 350 $\times 10^{-6}$ µkat. These data demonstrate that earthworms, in fact, possess active GST, but have only a limited capability to detoxify herbicidal compounds.

Induction of GST after herbicide stress – In order to study the abundance of *E. fetida* GST isoforms and their reaction to herbicide stress, GSTs were also extracted from animals exposed to herbicides for 24 and 48 hrs, respectively. The GST fraction after ammonium sulfate precipitation had similar activities as described in Table 1, but with specific differences (Fig. 1, see overleaf).

Both, CDBN and pNBC conjugation were stimulated extremely strongly after the treatments, without time dependency. Furthermore, the other minor GST activities for the conjugation of DCNB and PNBOC showed changes that were strongest after incubation with metolachlor, and the activity for PNBOC was even lost after 48 hrs. Such a loss of enzyme activity points to strong changes in the GST pattern as such (Table 2).

Table 2: Purification protocol of soluble protein from *Eisenia fetida*. Data represent means from at least three independent measurements $\pm$ SD

Control	Vol. (ml)	GST Volume Activity (µkat/ml)	Protein (mg)	GST Specific activity (µkat/mg)	GST Recovery (activity)	Purification	Recovery protein
Crude extract	10	10.3 $\pm$ 0.59	4.0 $\pm$ 0.028	2.5 $\pm$ 0.35	100.0%	1.00	100.0%
AmSO ₄ Precipitate	3.5	9.9 $\pm$ 1.83	2.7 $\pm$ 1.7	4.4 $\pm$ 1.4	96.8%	1.7	67.3%
Affinity Peak	11	2.7 $\pm$ 1.9	0.1 $\pm$ 0.006	375 $\pm$ 27.6	18.7%	147.1	3.0%

Fig. 1: Conjugation rates of earthworm GST with different substrates after exposure toward Fenoxaprop (FP) and Metolachlor (MC) herbicides for 24 and 48 hrs. CDBN conjugation is scaled on the left ordinate, the other substrates relate to the right y-axis. Separate controls (= untreated animals) have been assayed for both incubation studies. All experiments were repeated three to five times using ten and twenty animals per time point, respectively, and measurements were performed in triplicate. Rates are given in $\mu\text{kat mg protein}^{-1} \pm$ standard deviation

To obtain more information about the changes in enzyme activity under herbicide stress, we applied protein extracts to affinity chromatography. The soluble protein had been extracted from the animals as above, but a sophisticated purification on affinity chromatography matrices was subsequently performed.

With non-bound GST, eluted when the column was rinsed with buffer A, but after the addition of GSH in increasing concentrations in the presence of NaCl (buffer B), we found a rapid elution of GST in one peak. Main GST activity, as determined by CDBN, was found in fractions 14 to 24 of extracts from untreated animals (Fig. 2). It is worth mentioning that this method used for enzyme purification yielded GST at a recovery of 3.03% and a purification factor of 147-fold as compared to the crude extract. Purification factors between 50 and 200 after affinity chromatography are generally referred to as satisfactory in the literature (Schröder and Götzberger 1997).

When compared to treated animals, significant differences were observed in the elution of GST from the affinity matrices, firstly, the peak of non-bound GST, eluted earlier and more sharply than the same peak in the control experiment. Secondly, bound GST eluted in three distinguishable peaks, albeit at same amount of enzyme activities. This gave rise to the assumption that the GST isoenzyme pattern had changed during the incubation with the selected herbicides, either due to stress or as a defense reaction (see Fig. 2).

In this study, after affinity chromatography, the purified protein fraction should only contain enzymes related to glutathione and glutathione metabolism. Thus, further characterization via PAGE should yield information about the distribution of GST in this fraction.

When the main peaks from the affinity chromatography runs were spotted on acryl, amide gels and PAGE was performed,

Fig. 2: Elution of *Eisenia fetida* GST from GSH-Agarose affinity chromatography. A: Elution of GST from controls; B: GST pattern after 48 hrs Metolachlor (MC) treatment. Triangles: protein concentration, circles: GST activity per unit volume. Fractions 1 to 12 elution with buffer A, Fractions 13 to 25 elution with buffer B (increasing GSH concentration from 0 to 20 mM)

Fig. 3: Polyacrylamide gel electrophoresis of *Eisenia* GST after affinity chromatography. Bands: 0: MW standard; 1: untreated control; 2:FP-24h; 3:FP-48h; 4:MC-48h; 5:MC-24h; 6: untreated control. Abbreviations: Fenoxaprop (FP), Metolachlor (MC)

significant differences in the GST subunit patterns of controls and treatments were observed (Fig. 3).

GST subunits with an apparent molecular weight of 20 to 30 kDa migrate as distinct bands in the gels. Controls contained four bands at 25.3, 27.6, 28.5 and 29.5 kDa, but the samples treated with the herbicide fenoxaprop for 24 hrs, contained three bands at 26.6, 27.9 and 29.5 kDa. When samples of the 48h treatment period were investigated, we found that only two bands in the GST subunit range were left (26.6 and 27.9 kDa). Interestingly, the lightest and the heaviest bands (approximate MW 25.3 and 29.5 kDa) seemed to be lacking in the gel, although care had been taken to load the gels with equal amounts of protein. Metolachlor seemed to have further influence on the GST isoform distribution as only the central GST subunit bands (26.6 and 27.9 kDa) were found after 24 hrs of treatment. Such apparent losses of GSTs after treatment with xenobiotics have been reported in some cases (Pflugmacher and Schröder 1994, Schröder et al. 1992, Schröder and Götzberger 1997).

Visualization of glutathione conjugation. Such exposure to herbicides, although PAGE would indicate losses of GS subunits, can be shown to lead to significant changes in the formation of GS-conjugates in vivo. To test this hypothesis, earthworms were infiltrated with the fluorescent dye, Monochlorobimane (MCB), after pre-exposure to herbicides. MCB is conjugated to glutathione in the presence of GSTs. Fig. 4 shows in exposed earthworms that a strong fluorescence appears in the intersegments and the intestine, whereas the control animals do not develop significant fluorescence. Interestingly, the fenoxaprop treated group of earth worms developed the stron-

Fig. 4: Formation of fluorescent glutathione conjugates after exposure of earthworms to fenoxaprop. Fluorescence development was recorded after 2 hrs of incubation with Monochlorobimane (MCB). Upper left: control earthworms with MCB; Upper right: earthworms after preincubation with fenoxaprop and MCB, Middle: untreated control

gest fluorescence in the intestine of the animals, thus indicating an enhanced formation of the respective conjugate under the inductive influence of the herbicide.

3 Discussion

Earthworms which inhabit soils and litter layers in most landscapes can offer an important tool to evaluate environmental toxicity and impacts of organic xenobiotics and agrochemicals (Paoletti 1999, Hund-Rinke et al. 2003, Muthukaruppan et al. 2005). Pesticides can exert both direct toxicity against earthworms or produce latent effects on their growth, histology and fertility. In addition, pesticide-contaminated earthworms can present a source of contamination of higher members of the food web, e.g. birds or mammals. Pesticides usually enter the soil as residues of sprays applied to crop web plants from the above ground and are less frequently applied directly on soils as in the case of pre-emergence herbicides, nematocides or root pest control agents (Paoletti 1999). Upon entry into the soil, especially pesticide mixtures are expected to have strong effects on earthworms feeding at the soil surface (Paoletti 1999). In our short term experiments with the herbicides FP and MC, the earthworms did not develop visible damage, but they tried to avoid the substrate, in a similar manner as described by other authors (Hund-Rinke and Wiechering 2001, Hund-Rinke et al. 2003), after exposure to PAH and TNT metabolites.

So far, glutathione S-transferase activity has been demonstrated in 6 earthworm species of the family *Lumbricidae*, including *Eisenia fetida*. Considerable activity was obtained with the model substrate CDNB. As determined by enzymatic assay all the species contained an average of about 0.5 mmol/g fresh weight of glutathione (Stenersen et al. 1979). The author suggested that conjugation catalyzed by glutathione S-transferase may consequently be an important detoxification mechanism in earthworms (Stenersen et al. 1979). Subsequently, the role of glutathione S-transferase in the detoxification of xenobiotics by earthworms was studied (Stenersen 1984). GST activity was divided into 6 groups

after purification in *E. andrei* by Borgeraas and coworkers (1996). The isoforms of GST in this species displayed a different substrate specificity towards the substrates CDNB, DCNB, ethacrynic acid (ETHA) and cumene hydroperoxide. Our present study in *E. fetida* adds evidence that even more model compounds, amongst them the chemically-related p-nitrobenzylchloride (PNBC) and p-nitrobenz-oxylchloride (PNBOC) are also substrates for *E. fetida* GSTs. It is noteworthy, in our cultures, that DCNB conjugation was a hundred times lower than CDNB conjugation and that PNBC was by far the worst model substrate. Differences had also been reported concerning the subunit composition of GST, determined by SDS-PAGE and reversed phase HPLC. *E. andrei* GST 1V consisted of a protein involving subunit Ea 6, with an apparent mol wt. of 26.5 kD (Borgeraas et al. 1996). In the current study, the control GSTs from *E. fetida* contained three bands at 25.3, 27.6 and 29.5 kDa and the samples treated with the herbicides FP and MC again had a different pattern. Such changes in the isoenzyme pattern had also been observed in other organisms under heavy metal stress (Schröder et al. 2002) or after exposure to chlorinated acetic acids (Schröder et al. 1997).

In a study by Saint-Denis and coworkers (1998) on GST activity in *E. fetida*, the experimental conditions used were found to induce significant nonenzymatic conjugation rates higher than the enzymatic rates. Duration of exposure had a significant effect on the GST activities when *E. fetida* were exposed to different concentrations (120 and 250 mg/kg) of lead in artificial soil for 28 days. A recently published study on the effects of acetochlor on *Eisenia* resulted in very low changes in the CDNB conjugation after 60 days at concentrations of up to 80 mg/kg, whereas cellulose activity and growth were significantly diminished (Xiao et al. 2006).

Data obtained by Arnaud and co-workers (2000) showed that the biomarkers may vary significantly over a short period of 2 weeks in stock control worms, and that these changes could not be related to season, weather, temperature or any other stress. As can be deduced from Fig. 1, earthworms from our control plots did also show different GST values, but in any case, the GST activity after treatment with the herbicides was significantly higher than in untreated controls. Interestingly, the highest induction factors were observed with the rarely investigated GST substrates PNBC and PNBOC. The fact that PNBOC activity was lost after 48 hrs of incubation with MC might be even more important, as it shows the influence of a specific herbicide on a distinct group of GSTs responsible for the conjugation of this model compound. With view to the usefulness of GST as a biomarker, it might be speculated that the value and reliability of the GST enzyme test is also dependent on the substrate under consideration (Pflugmacher and Schröder 1994).

Stenersen and coworkers (1992) who have pointed to *E. andrei* and *E. fetida* have a high lethal threshold to carbamate pesticides. When earthworms were exposed to increasing concentrations of carbaryl (12, 25 and 50 mg/kg artificial soil) for different periods of time (2, 7, and 14 days) using the standard soil method, GST activity seemed to decrease after 2 and 14 days of exposure. Furthermore, the

study showed that carbaryl seemed to be a competitive inhibitor of biotransformation enzyme activities, but did not induce oxidative stress. As oxidative stress is commonly thought to be a major inducer of GSTs, this could explain the lack of GST induction. Instead, significant changes were detected in phase-one reactions and in acetylcholin-esterase activities upon exposure to lowest dose of carbaryl (Ribera et al. 2001).

Finally, the development of fluorescent GS-conjugates after stress is a novel observation, trying to bridge the problem of GST determination with various substrates and the plethora of environmental variables influencing GST activities (Schröder and Stampfl 1999). The accumulation of GS-conjugates and their determination via fluorescence microscopy is therefore a quick and secure marker for exposure that should be further developed to yield a reliable biotest.

4 Conclusions

Conjugation of several xenobiotic with model substances and the herbicides fenoxaprop and metolachlor has been proven in the earthworm, *E. fetida*. Former enzymological work had concentrated on GST activity with the substrate 1-chloro-2,4-dinitrobenzene. According to the authors' knowledge, here, for the first time, evidence is presented that *E. fetida* GST are also capable of conjugating a wider range of xenobiotic substrates including PNBOC, fenoxaprop and metolachlor. Different forms of GST were observed and changes in GST isoforms due to the treatment with selected herbicides were also noticed. GST conjugation rates varied between different herbicides used in this experiment. When treated worms were incubated with the fluorescent glutathione sensitive dye MCB, a strong fluorescence of the intestinal tract and the intersegments compared to untreated controls was observed, indicating that GST induction was organ specific.

In conclusion, it might be assumed that several herbicides may be detoxified by earthworms, to a small extent, but that they are strong stress factors influencing the detoxification system of the animal. High doses or long exposure might lead to deleterious effect on earthworms and limit their survival rate. The use of the animals as bioindicators for herbicides of different chemical classes (i.e. aryloxy propionic acids, chloroacetanilides) and their residues seems very promising, and a simple fluorescence test is presented.

5 Recommendations and Perspectives

The accumulation of GS-conjugates and their determination via fluorescence microscopy is a quick and secure, additional marker for exposure that should be further developed to complement existing biotests. The described methods and endpoints might help to understand the complex reaction of earthworms towards the herbicides tested and lead to an adapted test methodology.

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