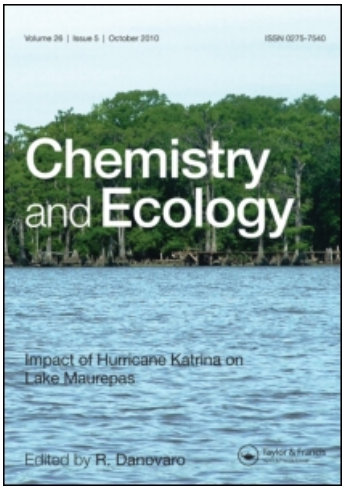

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Antioxidant and metabolic responses induced by cadmium and pyrene in the earthworm *Eisenia fetida* in two different systems: contact and soil tests

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Antioxidant and metabolic responses induced by cadmium and pyrene in the earthworm *Eisenia fetida* in two different systems: contact and soil tests

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The effects of cadmium and the polycyclic aromatic hydrocarbon (PAH) pyrene on the earthworm *Eisenia fetida* were investigated in contact and soil tests. Metabolic (glutathione-S-transferase, GST) and oxidative (catalase, CAT) stress enzymes were studied as biomarkers in earthworms after 48 hours, 14 days and 28 days. Contact test indicated that cadmium had significant effects on survival and enzyme activities while pyrene influenced neither in the studied concentrations. Induction of CAT and GST in earthworms exposed to cadmium and pyrene in the acute soil test (14 d) revealed the metabolism of these chemicals resulting in the production of reactive oxygen species. After a relatively long period of exposure (28 d), earthworms exposed to pyrene failed to handle the high toxicity and were physiologically damaged, while those exposed to cadmium adapted to the disturbed environment through effective metabolism of the chemical and management of the oxidative stress.

Keywords: cadmium; pyrene; glutathione-S-transferase (GST); catalase (CAT); earthworm (*Eisenia fetida*); contact test; soil test; acute and chronic exposure; OECD soil

1. Introduction

Biological indicators have been employed in ecological risk assessment as a supplement to chemical analysis, while choosing sensitive and ecologically relevant assays remains a key issue for the success of biological techniques [1–3]. In soil monitoring and contamination assessment, earthworms have been extensively studied as one of the most suitable bioindicator organisms [4,5] for their favourable effects on soil structure and function [6,7], representation for the majority of the total soil biomass [8,9] and continuous exposure to soil chemicals through their alimentary surfaces and skins [10–13], etc. Among several earthworm species, *Eisenia fetida* was recommended

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in OECD and ISO acute and chronic exposure protocols [14–16] and thus has been used most commonly in testing chemicals in soil.

In addition to toxicological endpoints, biomarkers could be used as diagnostic and prognostic early-warning tests to detect and assess the effects of pollution, particularly low concentrations of complex mixtures of contaminants, on environmental quality [17]. However, earthworm biomarkers have scarcely been investigated, particularly under field conditions, and provide an indication of exposure only [18]. Hankard (2004) found that it is ineffective to make a simple comparison of soil pollutant concentrations with guideline values in unequivocally identifying chemical exposure and toxic effect for soil organisms, and thus recommended applying earthworm biomarkers in indicating significant exposure and biological effects caused by a mixture of chemicals [2]. Also, biomarkers were recently integrated in an ecological weight-of-evidence (WOE) framework and thus gained ecotoxicological meaning [19].

In the potential biomarkers, GST and CAT were among the most studied because of their high sensitivity and ecological relevance. Both of the enzymes are involved in organisms' antioxidant mechanisms and can protect cells against adverse effects of reactive oxygen species (ROS) and xenobiotics [20–22]. CAT is responsible for breaking down the free radical by-product hydrogen peroxide into water and molecular oxygen [23]. GST catalyses the conjugation reaction of GSH and electrophilic xenobiotics, and thus contributes to the removal of reactive electrophiles [21]. Meanwhile, GST also serves as an important phase II detoxification enzyme to excrete and eliminate the products of phase I metabolism.

Heavy metals and PAHs are both common soil contaminants with high persistence and toxicity. In fact, they are frequently found together in soils [24]. Findings of previous work have confirmed this result and ignited our interest in these two categories of chemicals, which might have some similarities in sources of release, detoxification mechanisms and metabolic dynamics [25–28].

Cadmium is a heavy metal of considerable environmental and occupational concern because of its carcinogenicity in humans and rodents [29–32]. While the potential mechanism(s) of cadmium carcinogenicity is unknown, it does produce oxidative stress [33]. Various studies have shown that cadmium induces the production of hydroxyl radicals [34], superoxide anions, nitric oxide and hydrogen peroxide [35,36]. In earthworms, some studies have shown that cadmium could affect lysosomal membrane stability, metallothionein gene expression and lysosome-associated-glycoprotein gene expression, in the laboratory [37]. Li et al. (2009) investigated cadmium-induced effects on protein content, cellulase activity and DNA damage in the earthworm *E. fetida* [38]. Yet few have discussed the effects of cadmium on the earthworm's antioxidant and metabolic enzymes.

PAHs can be released into the environment through, for example, oil and gas spills, atmospheric deposition, sewage sludge application, or pollution from old gas works or sites used for drying tar-coated fishing nets [39]. Among PAHs, pyrene is considered to be non-carcinogenic by the International Agency for Research on Cancer and the US Environmental Protection Agency [20]. While there are intensive investigations into B[a]P toxicity because of its known carcinogenicity, only a few research studies have been conducted to assess pyrene toxicity in earthworms. Brown et al. (2004) investigated pyrene toxicity in the earthworm *Lumbricus rubellus*, and an LC_{50} of $283\,mg\,kg^{-1}$ in artificial soil tests after a 7-day exposure was reported [20]. On the sub-lethal level, it is thought that the metabolism of PAHs by cytochrome P4501a will produce free radicals, which the organism might activate the antioxidant system to cope with. Yet very little effort was made to discuss this response in earthworms.

All these considerations lead to the hypothesis that cadmium and pyrene might have separate and/or combined effects on oxidative stress defense and biotransformation systems in earthworms. To explore the potential combined effects, we would like to start in this study to firstly discuss antioxidant and metabolic responses of the earthworms exposed to cadmium and pyrene separately

in contact and acute and soil tests after acute and chronic exposure. GST and CAT activities are selected as sub-lethal biological endpoints to indicate potential effects on antioxidant and metabolic systems.

2. Methods

2.1. *Earthworm exposure protocol*

The earthworm *Eisenia fetida* and OECD soil were employed to generate comparable results. The earthworm *Eisenia fetida* was purchased from an earthworm farm in Beijing, maintained in an earthworm culture according to the specifications of the OECD protocol and kept in the dark at $20 \pm 1°C$ [14]. Adult worms with well-developed clitellum and an individual weight around 350 mg were picked up and kept on moist filter paper for 3 h to avoid gut contents. They were then rinsed and dried before all exposure tests.

Contact filter paper tests were conducted according to the OECD protocol [14]. Flat-bottomed glass vials (8 cm × 3 cm) were employed and their sides lined with filter paper without overlapping to achieve an exposure area of around 65 cm^2. The toxicity of cadmium and pyrene was assessed using 10 earthworms in negative controls and at four logarithmic concentrations from 0.0001–0.1 mg/cm^2. Cadmium chloride ($CdCl_2.2.5H_2O$) (Sigma-Aldrich, Beijing, China) was dissolved in deionised water, and pyrene (Sigma-Aldrich, Beijing, China) in acetone, to obtain the required dilution series. Controls were exposed to 1 ml of deionised water or acetone only. 1 ml of the required dilution was added to the filter paper and left to evaporate for 24 h. Filter papers were rehydrated with 1 ml of deionised water; one earthworm placed and each vial sealed with plastic film with a small ventilation hole. The tests were conducted in the dark at $20 \pm 1°C$. After 48 h earthworms still alive were counted, snap-frozen in liquid nitrogen and stored at $-80°C$ until use.

Artificial soil tests were conducted using OECD soil [14] with two exposure periods: 14 and 28 days. An appropriate amount of pyrene was dissolved in 50 ml acetone (acetone only in solvent controls), cadmium in 50 ml deionised water (water only in controls), and each dilution mixed with 750 g soils carefully with stainless tools to obtain media containing 40, 80, 160, 640, 1280 mg pyrene kg^{-1} soil and 50, 100, 200, 400, 800 mg cadmium kg^{-1} soil. All the soils spiked with acetone were left in a well ventilated fume hood and turned to allow the acetone to evaporate. The soils were then rehydrated to give an overall moisture content of about 35% of the final weight [40], mixed thoroughly, and left for one day to equilibrate [20]. Soil pH was checked as described by ISO 10390 (1998) and adjusted to 6.0 ± 0.5 by addition of calcium carbonate [14]. All exposures were conducted in one-litre glass jars at $20 \pm 1°C$ under continuous light to ensure that the earthworms remained burrowed in the soil [40]. For each duration and dose condition, four replicates, consisting of ten worms pooled together, were tested. The moisture content of each replicate was checked weekly and water added as required. During the tests, no foods were supplemented and mortality was assessed after 14 and 28 days. At the end of the test period, survivors were nap-frozen in liquid nitrogen and stored at $-80°C$ until use.

2.2. *Sample preparation*

All procedures were carried out at 4°C. Samples were frozen at $-80°C$ until use.

Three individuals from each replicate – at most 12 for each treatment – were picked up randomly and ground in liquid nitrogen two by two as a pool to produce three to six samples for each treatment. Samples were then thawed on ice and homogenised in TRIS buffer (100 mM, pH 7.5)

in a 0.25 w/v ratio at 4°C for 1 min at 12,000 rpm. The homogenate was centrifuged at 9000 g for 30 min to create the post-mitochondrial fraction (supernatant: S9). Aliquots were frozen at −80°C until use.

2.3. *Biological assays*

CAT activity was measured as described in Claiborne (1985) and Saint-Denis et al. (1998, 2001) [23,40,41]. The variation of absorbance at 240 nm due to the dismutation of H_2O_2 was measured (absorbance 40 $M^{-1}cm^{-1}$). Assay conditions were as follows: 67 mM Tris buffer, pH 7.4, 15 mM H_2O_2 and 100 μg sample proteins in 3 ml incubate. Incubation was carried out at 25°C and the reaction was initiated by the addition of H_2O_2.

GST activity was measured using the method described in Habig et al. (1974) and Saint-Denis et al. (1998, 2001) [40–42]. The assay was conducted by monitoring the appearance of the conjugated complex of CDNB and GSH (absorbance 9.6 m $M^{-1}cm^{-1}$) at 340 nm over time. Assay conditions were as follows: 100 mM Tris buffer, pH 7, 2 mM GSH, 1 mM CDNB, 100 μg sample proteins (only buffer for the spontaneous reaction), and 3 ml incubate. The reaction was conducted at 25°C and initiated by the addition of GSH.

Protein content was assayed according to Bradford (1976) with BSA as the reference substance [43].

2.4. *Statistics*

Parametric tests were preceded by tests for normal distribution and for homogeneity of variances. Non-homogeneous data were transformed appropriately. Only when both preconditions are met, differences in enzyme activities were tested for treatment significance using one-way analysis of variance (ANOVA). Whenever a significant effect was detected, post-hoc comparison (LSD test) was conducted to check differences between exposed and control groups. All statistical analysis was performed using the SPSS program (Standard Version 13.0, SPSS Inc.).

3. Results and discussion

3.1. *48-hour contact paper test*

Survival of the contact paper tests is presented in Table 1. All worms survived in the (solvent) controls and at all pyrene concentrations, while no survivors were found at the two highest concentrations of cadmium, indicating that cadmium had a significant effect on worms' survival, while pyrene did not in the studied dose range.

Cadmium had a significant effect on CAT activity ($F = 8.378$, $p = 0.006$). Post-hoc comparison indicated that both GST ($p < 0.01$, Figure 1) and CAT ($p < 0.01$, Figure 2) activities were significantly higher at the highest sub-lethal concentration (0.001 mg/cm^2) of cadmium. Pyrene affected neither of the enzyme activities in the tested concentrations.

The paper contact test is thought to be a screening test and provides limited information about the real situation in the field. Yet it indeed helps to plan and arrange further experiments. In the present study, cadmium had significant effects on survival and on both of the enzymes at the highest sub-lethal concentration in the contact test, indicating a feasible test in soils in the next step. As for pyrene, though no change of either enzyme activity was observed, a soil test was still carried out because all worms survived at the highest concentration, which might indicate a higher dose of effect for pyrene beyond the chosen concentrations.

Table 1. Survival of *Eisenia fetida* exposed to control, solvent control and four concentrations of pyrene and cadmium separately in a contact filter paper test for 48 h.

Pyrene concentration, mg/cm^2	Cadmium concentration, mg/cm^2	Survival, %
Control		100
Solvent control		100
10^{-4}	–	100
10^{-3}	–	90
10^{-2}	–	90
10^{-1}	–	100
–	10^{-4}	100
–	10^{-3}	80
–	10^{-2}	0
–	10^{-1}	0

Note: Values are percentage survival for 10 worms kept individually in treated vial.

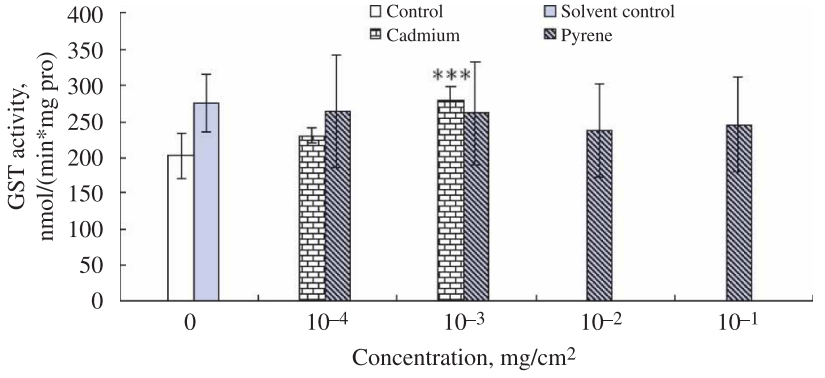

Figure 1. GST activity of the earthworm *Eisenia fetida* exposed to four concentrations of cadmium and pyrene separately for 48 h in the contact paper test. Bars are the mean of 3–5 replicates (± SD), with each replicate being the mean of two worms (if available), except at the highest two concentrations of cadmium, where no survivors were found. Note: *Significantly different from control at $p < 0.05$. ***Significantly different from control at $p < 0.01$.

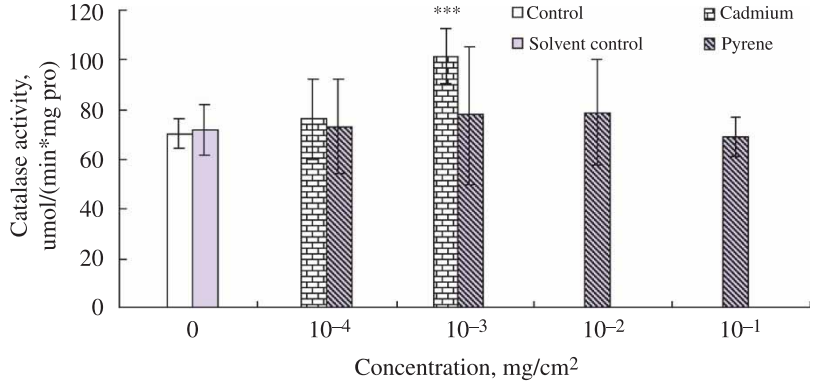

Figure 2. CAT activity of the earthworm *Eisenia fetida* exposed to four concentrations of cadmium and pyrene separately for 48 h in the contact paper test. Bars are the mean of 3–5 replicates (± SD), with each replicate being the mean of two worms (if available), except at the highest two concentrations of cadmium, where no survivors were found. Note: *Significantly different from control at $p < 0.05$. ***Significantly different from control at $p < 0.01$.

3.2. *14-day artificial soil test*

GST and CAT activities in whole worms after 14-day and 28-day exposures in soil tests are presented in Figure 3 and Figure 4. Cadmium ($F = 51.631$, $p = 0.000$) and pyrene ($F = 3.384$, $p = 0.028$) both had significant effects on GST activity after 14 days. Post-hoc comparison indicated that GST activity was significantly increased at 100, 200, 400 mg/kg cadmium ($p < 0.01$) and at the highest sub-lethal concentration of pyrene (320 mg/kg) ($p < 0.05$).

In the soil tests, the level of GST was comparable to that found in *Eisenia foetida* [44] and *Pheretima posthuma* [45] exposed to pesticides and in *Lampito mauritii* exposed to Pb [21]. GST activity was significantly induced at all concentrations of cadmium, except at the lowest (50 mg/kg), and at the highest concentration (320 mg/kg) of pyrene after 14 days, revealing the metabolism of each of the chemicals resulting in the production of ROS in acute exposure. Evidence of the functioning antioxidant defense against the cellular ROS reactions by GST under metal exposure was clearly seen in the field and laboratory results. GST activity increased with decreasing distance to the emission source and with increasing soil metal concentrations in the laboratory [46]. GST response to pyrene exposure was barely reported to date, while Saint-Denis et al. (1999) found no effect of B[a]P on GST activity in *Eisenia fetida andrei* after 1–14 days [47].

Cadmium ($F = 6.962$, $p = 0.001$) and pyrene ($F = 9.591$, $p = 0.000$) both had significant effects on CAT activity after 14 days. Post-hoc comparison indicated that CAT activity was significantly increased at the highest sub-lethal concentration of cadmium (400 mg/kg) ($p < 0.01$), and at 160 and 320 mg/kg pyrene ($p < 0.01$).

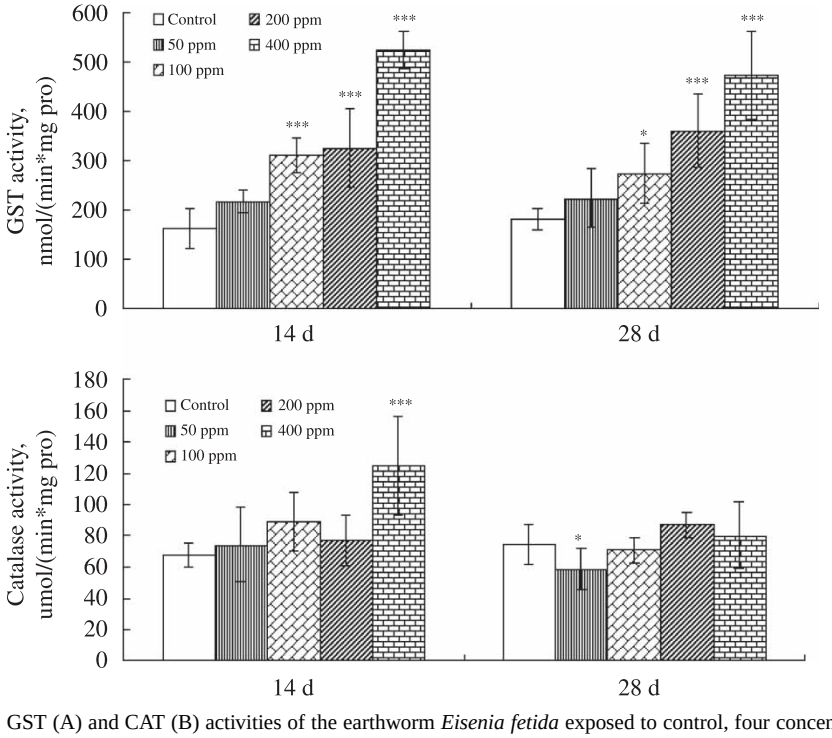

Figure 3. GST (A) and CAT (B) activities of the earthworm *Eisenia fetida* exposed to control, four concentrations of cadmium for 14 and 28 days. Values are means (± SD) of six replicates per treatment, with each replicate being the mean of two worms.
Note: *Significantly different from control at $p < 0.05$. ***Significantly different from control at $p < 0.01$.

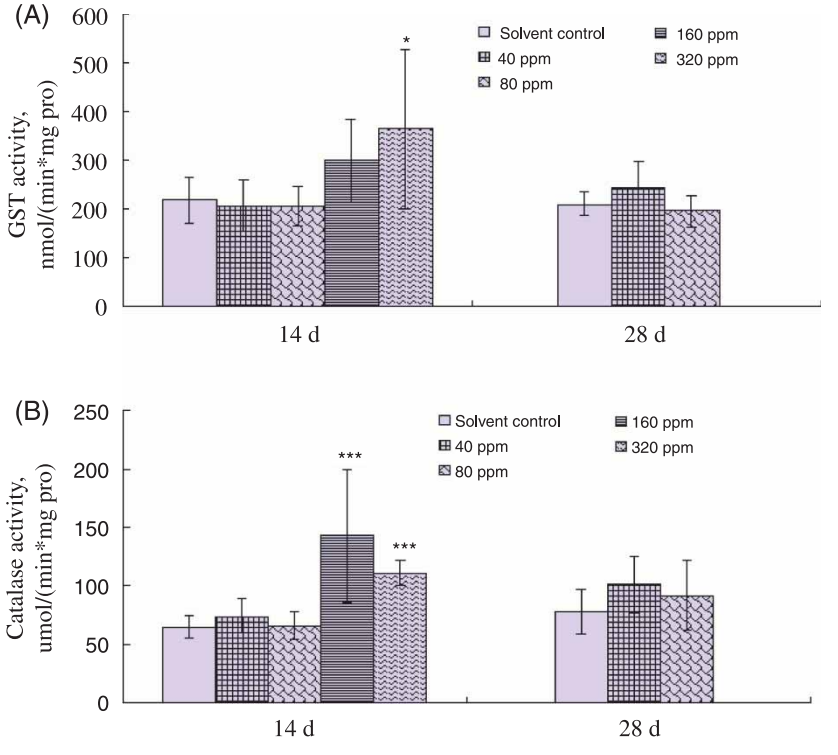

Figure 4. GST (A) and CAT (B) activities of the earthworm *Eisenia fetida* exposed to solvent control, four concentrations of pyrene for 14 and 28 days. Values are means (± SD) of six replicates per treatment, with each replicate being the mean of two worms (if available), except at 320 mg/kg pyrene after 14 days, where only six survivors were found, and at 160 and 320 mg/kg pyrene after 28 days, where no worms survived.
Note: *Significantly different from control at $p < 0.05$. ***Significantly different from control at $p < 0.01$.

The level of CAT was comparable to that reported in *Lumbricus rubellus* exposed to pyrene [20] and *Eisenia fetida andrei* exposed to carbaryl [44]. Both cadmium and pyrene could activate CAT after acute exposure. CAT activity was significantly induced at the highest concentration (400 mg/kg) of cadmium and the highest two concentrations (160, 320 mg/kg) of pyrene, increasing around 2-fold compared with that of control. The increase of CAT activity may be stimulated by hydrogen peroxide produced in the biotransformation and metabolism of these chemicals. There are many reports about the effects of cadmium on cellular antioxidant enzymes *in vitro* and *in vivo* (rats). Short-term exposure to cadmium has been shown to decrease the activities of almost all of these enzymes, whereas with more elevated doses and extended exposure enhancement of activities – including CAT [48–50] and GST [49] – was also found, probably because of adaptive induction of genes [51]. On the other hand, Saint-Denis et al. (1999) found B[a]P can induce CAT activity at certain concentrations (0.05, 100 and 1000 mg kg^{-1}) in soil tests after a 7-day exposure for *Eisenia fetida andrei* [47]. Actually, the ability of earthworms to biotransform and excrete PAHs is crucial for their survival in the presence of these both carcinogenic and narcotic chemicals [52].

3.3. *28-day artificial soil test*

After 28 days, cadmium ($F = 19.715$, $p = 0.000$) still had a significant effect on GST activity. Post-hoc comparison indicated that GST activity was significantly increased at 100 ($p < 0.05$),

200 ($p < 0.01$), and 400 ($p < 0.01$) mg/kg cadmium. Also, cadmium had a significant effect on CAT activity ($F = 3.742$, $p = 0.016$). Post-hoc comparison indicated that CAT activity was significantly inhibited at 50 mg/kg cadmium ($p < 0.05$). For both exposure periods, GST responses to cadmium exposure seem to be dose dependent.

For pyrene-spiked treatments, no survivors were found in treatments in which significant enzyme activity variation was previously detected. And pyrene had no effect on either of the enzyme activities in other treatments after 28 days.

Maity et al. (2008) found that GST activity can be significantly induced in *Lampito mauritii* under Pb exposure after 2 and 7 days, while it fell down to the control lever after 14 days [21]. A similar result was found by Saint-Denis et al. (2001), in which in the earthworm *Eisenia fetida andrei*, GST activity was already decreased after 2 days' exposure to lead acetate [40]. Yet, it was also reported that GST activity could be still increased in *Eisenia fetida* after as long as 45 and 60 days under exposure to herbicide acetochlor [53]. In the present study, the significant induction (3-fold) of GST activity with a relatively long duration of the effect (28 days) under exposure to cadmium, indicated that phase II detoxification mechanism was still activated to metabolise the metal, and/or that the earthworm might not possess other detoxification mechanisms and/or a phenotypic adaptive mechanism [54].

After 28 days, CAT activity at 400 mg/kg cadmium had been stabilised back to the level of control, showing that the worms had adapted to the environment disturbed by the presence of cadmium. Yet at 160 and 320 mg/kg pyrene no survivors were found, indicating the worms failed to cope with the toxic stress from exposure to pyrene through their detoxifying and antioxidant mechanisms during a relatively long exposure period.

Brown et al. (2004) reported that CAT activity could be inhibited in the earthworm *L. rubellus* at 640 mg/kg pyrene after 42 days [20]. In contrast, no change of CAT activity was found at the lowest two concentrations (40, 80 mg/kg) of pyrene treatments after 28 days in this study. This could be attributed to the different dose and duration of effect and sensitivity to pyrene of these two species. Nonetheless, at 50 mg/kg cadmium, where no change of CAT activity was previously detected, the enzyme was significantly inhibited after 28 days. The inhibitory effect on CAT activity after cadmium exposure is possibly due to cellular dysfunction or apoptosis caused by the toxic stress [55]. According to Cossu (1997), pollution involves an induction of antioxidant enzymes allowing the organisms to partially or totally overcome stress resulting from exposure to an unsafe environment [56]. However, too great toxicity involves their inhibition, although the CAT activity decrease could be explained too by the presence of the ROS. Here, when the system works correctly, superoxide dismutase catalyses the dismutation of superoxide into oxygen and hydrogen peroxide. But, when the quantity of ROS is too great, they become CAT inhibitors [57,58], i.e. the severe toxic stress implies insufficient metabolism [59]. Similar results were reported by Brulle et al. (2006) that after exposure to cadmium (80 and 800 ppm), CAT mRNA expression levels showed a tendency with an induction at the beginning followed by an inhibition as the exposure time extended [60].

4. Conclusion

We validated the response of antioxidant and metabolic systems of the earthworm *Eisenia fetida* as a sign of adaptation to disturbed terrestrial ecosystems. Induction of GST and CAT activities in the earthworm *Eisenia fetida* exposed to cadmium and pyrene after 14 days indicated that the metabolism of each of the chemicals produced ROS in acute exposure. After 28 days, enzyme activities were stabilised at the control level or even inhibited, except for cadmium-spiked treatments, where GST activity was significantly increased, indicating that the phase II detoxification

mechanism was still activated. By contrast, the earthworm failed to handle the toxic stress caused by pyrene in chronic exposure.

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References

[1] J. Cortet, A. Gomot-De Vauflery, N. Poinsot-Balaguer, L. Gomot, C. Texier, and D. Cluzeau, *The use of invertebrate soil fauna in monitoring pollutant effects*, Eur. J. Soil Biol. 35 (1999), pp. 115–134.
[2] P.K. Hankard, C. Svendsen, J. Wright, C. Wienberg, S.K. Fishwick, D.J. Spurgeon and J.M. Weeks, *Biological assessment of contaminated land using earthworm biomarkers in support of chemical analysis*, Sci. Total Environ. 330 (2004), pp. 9–20.
[3] J.E. Kammenga, R. Dallinger, M.H. Donker, H.R. Kohler, V. Simonsen, R. Triebskorn, and J.M. Weeks, *Biomarkers in terrestrial invertebrates for ecotoxicological soil risk assessment*, Rev. Environ. Contam. Toxicol. 164, (2000), pp. 93–147.
[4] C.A. Callahan, *Earthworms as ecotoxicological assessment tools*, in *Earthworms in Waste and Environmental Assessment*, C.A. Edwards and E.F. Neuhauser, eds., SPB Academic Publishing, The Hague, 1988, pp. 295–301.
[5] G.C. Goats, C.A. Edwards, *Prediction of field toxicity of chemicals to earthworms by laboratory methods*, in *Earthworms in Waste and Environmental Assessment*, C.A. Edwards and E.F. Neuhauser, eds., Academic Publishing, The Hague, 1988, pp. 283–294.
[6] A.G. Jongmans, M.M. Pulleman, M. Balabane, F. van Oort and J.C.Y. Marinissen, *Soil structure and characteristics of organic matter in two orchards differing in earthworm activity*, Appl. Soil Ecol. 24 (2003), pp. 219–232.
[7] M.G. Paoletti, *The role of earthworms for assessment of sustainability and as bioindicators*, Agric. Ecosyst. Envir. 74 (1999), pp. 137–155.
[8] A. Bouche, *Earthworm species and ecotoxicological studies*, in *Ecotoxicology of Earthworms. Intercept*, P.W. Greig-Smith, H. Becker, P.J. Edwards, F. Heimbach, eds., Andover, UK, 1992, pp. 20–35.
[9] A.M.A. Rida, *Les vers de terre et l'environnement*, La Recherche, 25 (1994), pp. 260–267.
[10] T. Jager, R.H.L.J. Fleuren, E.A. Hogendoorn and G. De Korte, *Elucidating the routes of exposure for organic chemicals in the earthworm*, Eisenia andrei (*Oligochaeta*), Environ. Sci. Technol. 37 (2003), pp. 3399–3404.
[11] A.J. Morgan, S.R. Sturzenbaum, C. Winters, G.W. Grime, N.A. Abd Aziz and P. Kille, *Differential metallothionein expression in earthworm* (Lumbricus rubellus) *tissues*, Ecotoxicol. Environ. Safety 57 (2004), pp. 11–19.
[12] J.K. Saxe, C.A. Impellitteri, W.J.G.M. Peijnenburg and H.E. Allen, *Novel model describing trace metal concentrations in the earthworm*, Eisenia andrei, Environ. Sci. Technol. 35 (2001), pp. 4522–4529.
[13] M.G. Vijver, H.T. Wolterbeek, J.P.M. Vink and C.A.M. van Gestel, *Surface adsorption of metals onto the earthworm* Lumbricus rubellus *and the isopod* Porcellio scaber *is negligible compared to absorption in the body*, Sci. Total Environ. 340 (2005), pp. 271–280.
[14] OECD, Organization for Economic Co-operation and Development, *Test 207: Earthworm acute toxicity tests*, in *OECD Guidelines for Testing of Chemicals*, Organization for Economic Co-operation and Development, ed., 1984.
[15] ISO, *Standard number No. 11268-2. Soil quality: Effects of pollutants on earthworms* (Eisenia fetida), *Part II: Method for the determination of effects on reproduction*, International Standard Organization, Geneva, Switzerland, 1998.
[16] OECD, Organization for Economic Co-operation and Development, *Test 222: Earthworm reproduction tests*, in *OECD Guidelines for Testing of Chemicals*, Organization for Economic Co-operation and Development, ed., 2004.
[17] D.R. Livingstone, *Biotechnology and pollution monitoring – use of molecular biomarkers in the aquatic environment*, J. Chem. Technol. Biotechnol. 57 (1993), pp. 195–211.
[18] J.C. Sanchez-Hernandez, *Earthworm biomarkers in ecological risk assessment*, Rev. Environ. Contam. Toxicol. 188 (2006), pp. 85–126.
[19] T. Neuparth, A.D. Correia, F.O. Costa, G. Lima, M.H. Costa, *Multi-level assessment of chronic toxicity of estuarine sediments with the amphipod* Gammarus locusta*: I. Biochemical endpoints*, Mar. Environ. Res. 60 (2005), pp. 69–91.
[20] P.J. Brown, S.M. Long, D.J. Spurgeon, C. Svendsen, and P.K. Hankard, *Toxicological and biochemical responses of the earthworm* Lumbricus rubellus *to pyrene, a non-carcinogenic polycyclic aromatic hydrocarbon*, Chemosphere 57 (2004), pp. 1675–1681.
[21] S. Maity, S. Roy, S. Chaudhury, and S. Bhattacharya, *Antioxidant responses of the earthworm* Lampito mauritii *exposed to Pb and Zn contaminated soil*, Environ. Pollut. 151 (2008), pp. 1–7.
[22] A. Rannug and U. Rannug, *Enzyme inhibition as possible mechanism of the mutagenicity of dithiocarbamic acid derivatives in* Salmonella typhimurium, Chemico-Biol. Interact. 49 (1984), pp. 329–340.
[23] A. Claiborne, *Catalase activity*, in *Handbook of Methods for Oxygen Research*, R. A. Greenwald, ed., CRC Press, Boca Raton, FL, 1985, pp. 283–284.

[24] J. Ahtiainen, R. Valo, M. Jarvinen, and A. Joutti, *Microbial toxicity tests and chemical analysis as monitoring parameters at composting of creosote-contaminated soil*, Ecotoxicol. Environ. Safety 53 (2002), pp. 323–329.
[25] G.Q. Shen, Y.T. Lu and J.B. Hong, *Combined effect of heavy metals and polycyclic aromatic hydrocarbons on urease activity in soil*, Ecotoxicol. Environ. Safety 63 (2006), pp. 474–480.
[26] N. Irha, J. Slet, and V. Petersell, *Effect of heavy metals and PAH on soil assessed via dehydrogenase assay*, Envir. Int. 28 (2003), pp. 779–782.
[27] G.Q. Shen, Y.T. Lu, Q.X. Zhou, and J.B. Hong, *Interaction of polycyclic aromatic hydrocarbons and heavy metals on soil enzyme*, Chemosphere 61 (2005), pp. 1175–1182.
[28] H.W. Mielke, E.T. Powell, A. Shah, C.R. Gonzales, and P.W. Mielke, *Multiple metal contamination from house paints: Consequences of power sanding and paint scraping in New Orleans*, Environ. Health Perspec. 109 (2001), pp. 973–978.
[29] S. Satarug, J.R. Baker, S. Urbenjapol, M. Haswell-Elkins, P.E.B. Reilly, D.J. Williams, and M.R. Moore, *A global perspective on cadmium pollution and toxicity in non-occupationally exposed population*, Toxicol. Lett. 137 (2003), pp. 65–83.
[30] M.P. Waalkes, *Cadmium carcinogenesis in review*, J. Inorg. Biochem. 79 (2000), pp. 241–244.
[31] M.P. Waalkes, M.R. Anver, and B.A. Diwan, *Chronic toxic and carcinogenic effects of oral cadmium in the noble (NBL/Cr) rat: Induction of neoplastic and proliferative lesions of the adrenal, kidney, prostate, and testes*, J. Toxicol. Environ. Health – Part A 58 (1999), pp. 199–214.
[32] M.P. Waalkes and R.R. Misra, *Cadmium carcinogenicity and genotoxicity*, in *Toxicology of Metals*, L. Chang, ed., CRC Press, Boca Raton, FL, 1996, pp. 231–244.
[33] P.L. Goering, M.P. Waalkes, C.D. Klaassen, *Toxicology of cadmium*, in *Handbook of Experimental Pharmacology: Toxicology of Metals, Biochemical Effects, Vol. 115*, R.A. Goyer and M.G. Cherian, eds., Springer-Verlag, New York, 1994, pp. 189–214.
[34] P. O'Brien and H.J. Salacinski, *Evidence that the reactions of cadmium in the presence of metallothionein can produce hydroxyl radicals*, Arch. Toxicol. 72 (1998), pp. 690–700.
[35] A. Galan, L. Garcia-Bermejo, A. Troyano, N.E. Vilaboa, C. Fernandez, E. de Blas and P. Aller, *The role of intracellular oxidation in death induction (apoptosis and necrosis) in human promonocytic cells treated with stress inducers (cadmium, heat, X-rays)*, Eur. J. Cell Biol. 80 (2001), pp. 312–320.
[36] S.J. Stohs, D. Bagchi, E. Hassoun, and M. Bagchi, *Oxidative mechanisms in the toxicity of chromium and cadmium ions*, J. Environ. Pathol. Toxicol. Oncol. 20 (2001), pp. 77–88.
[37] D.J. Spurgeon, S.R. Sturzenbaum, C. Svendsen, P.K. Hankard, A.J. Morgan, J.M. Weeks, and P. Kille, *Toxicological, cellular and gene expression responses in earthworms exposed to copper and cadmium*, Comp. Biochem. Physiol. C – Toxicol. Pharmacol. 138 (2004), pp. 11–21.
[38] M. Li, Z.T. Liu, Y. Xu, Y.B. Cui, D.S. Li and Z.M. Kong, *Comparative effects of Cd and Pb on biochemical response and DNA damage in the earthworm* Eisenia fetida (*Annelida, Oligochaeta*), Chemosphere 74 (2009), pp. 621–625.
[39] J. Jensen and L.E. Sverdrup, *Polycyclic aromatic hydrocarbon ecotoxicity data for developing soil quality criteria*, Rev. Environ. Contam. Toxicol. 179 (2003), pp. 73–97.
[40] M. Saint-Denis, J.F. Narbonne, C. Arnaud, and D. Ribera, *Biochemical responses of the earthworm* Eisenia fetida andrei *exposed to contaminated artificial soil: Effects of lead acetate*, Soil Biol. Biochem. 33 (2001), pp. 395–404.
[41] M. Saint-Denis, F. Labrot, J.F. Narbonne, and D. Ribera, *Glutathione, glutathione-related enzymes, and catalase activities in the earthworm* Eisenia fetida andrei, Arch. Environ. Contam. Toxicol. 35 (1998), pp. 602–614.
[42] W.H. Habig, M.J. Pabst, and W.B. Jakoby, *Glutathione-S-transferase: The first enzymatic step in mercapturic acid formation*, J. Biol. Chem. 249 (1974), pp. 7130–7139.
[43] M.M. Bradford, *A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding*, Anal. Biochem. 72 (1976), pp. 248–254.
[44] D. Ribera, J.F. Narbonne, C. Arnaud, and M. Saint-Denis, *Biochemical responses of the earthworm* Eisenia fetida andrei *exposed to contaminated artificial soil, effects of carbaryl*, Soil Biol. Biochem. 33 (2001), pp. 1123–1130.
[45] R.K. Hans, M.A. Khan, M. Farooq, and M.U. Beg, *Glutathione-S-transferase activity in an earthworm (Pheretima-Posthuma) exposed to 3 insecticides*, Soil Biol. Biochem. 25 (1993), pp. 509–511.
[46] T. Lukkari, M. Taavitsainen, M. Soimasuo, A. Oikari, and J. Haimi, *Biomarker responses of the earthworm* Aporrectodea tuberculata *to copper and zinc exposure: Differences between populations with and without earlier metal exposure*, Environ. Pollut. 129 (2004), pp. 377–386.
[47] M. Saint-Denis, J.F. Narbonne, C. Arnaud, E. Thybaud, and D. Ribera, *Biochemical responses of the earthworm* Eisenia fetida andrei *exposed to contaminated artificial soil: Effects of benzo(a)pyrene*, Soil Biol. Biochem. 31 (1999), pp. 1837–1846.
[48] M.M. Kostic, B. Ognjanovic, S. Dimitrijevic, R.V. Zikic, A. Stajn, G.L. Rosic, and R.V. Zivkovic, *Cadmium-induced changes of antioxidant and metabolic status in red-blood-cells of rats* – in-vivo *effects*, Eur. J. Haematol. 51 (1993), pp. 86–92.
[49] S. Sarkar, P. Yadav, and D. Bhatnagar, *Lipid peroxidative damage on cadmium exposure and alterations in antioxidant system in rat erythrocytes: A study with relation to time*, Biometals 11 (1998), pp. 153–157.
[50] A. Shukla, G.S. Shukla, and R.C. Srimal, *Cadmium-induced alterations in blood-brain barrier permeability and its possible correlation with decreased microvessel antioxidant potential in rat*, Human Exper. Toxicol. 15 (1996), pp. 400–405.
[51] M. Waisberg, P. Joseph, B. Hale, and D. Beyersmann, *Molecular and cellular mechanisms of cadmium carcinogenesis*, Toxicology 192 (2003), pp. 95–117.

[52] D.L. Milligan, J.G. Babish, E.F. Neuhauser, *Noninducibility of cytochrome P450 in the earthworm* Dendrobaena veneta, Comp. Biochem. Physiol. 85C (1986), pp. 85–87.
[53] N.W. Xiao, B. Jing, F. Ge, and X.H. Liu, *The fate of herbicide acetochlor and its toxicity to* Eisenia fetida *under laboratory conditions*, Chemosphere 62 (2006), pp. 1366–1373.
[54] L. Posthuma and N.M. van Straalen, *Heavy metal adaptation in terrestrial invertebrates: A review of occurrence, genetics, physiology and ecological consequences*, Comp. Biochem. Physiol. 100C (1993), pp. 11–38.
[55] L.I. Sweet, D.R. Passino-Reader, P.G. Meier, and G.M. Omann, *Xenobiotic-induced apoptosis: Significance and potential application as a general biomarker of response*, Biomarkers 4 (1999), pp. 237–253.
[56] C. Cossu, A. Doyotte, M.C. Jacquin, M. Babut, A. Exinger, and P. Vasseur, *Glutathione reductase, selenium-dependent glutathione peroxidase, glutathione levels, and lipid peroxidation in freshwater bivalves,* Unio tumidus, *as biomarkers of aquatic contamination in field studies*, Ecotoxicol. Environ. Safety 38 (1997), pp. 122–131.
[57] F. Geret, A. Serafim, L. Barreira, and M.J. Bebianno, *Effect of cadmium on antioxidant enzyme activities and lipid peroxidation in the gills of the clam* Ruditapes decussatus, Biomarkers 7 (2002), pp. 242–256.
[58] Y. Kono and I. Fridovich, *Superoxide radical inhibits catalase*, J. Biol. Chem. 257 (1982), pp. 5751–5754.
[59] E. Schreck, F. Geret, L. Gontier, and M. Treilhou, *Neurotoxic effect and metabolic responses induced by a mixture of six pesticides on the earthworm* Aporrectodea caliginosa nocturna, Chemosphere 71 (2008), pp. 1832–1839.
[60] F. Brulle, G. Mitta, C. Cocquerelle, D. Vieau, S. Lemiere, A. Lepretre, and F. vanden Bulcke, *Cloning and real-time PCR testing of 14 potential biomarkers in* Eisenia fetida *following cadmium exposure*, Environ. Sci. Technol. 40 (2006), pp. 2844–2850.