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Rank-based biomarker index to assess cadmium ecotoxicity on the earthworm *Eisenia andrei*

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HIGHLIGHTS

- We used a multi-biomarker approach on earthworms exposed to artificial Cd soils.
- The selected biomarkers have been integrated into the rank-based index.
- Not all biomarkers responded to Cd contamination in the same way.
- The rank-based index of 100 ppm Cd treatment resulted almost double compared to the control.
- Several biomarkers have shown significant correlation between them.

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ABSTRACT

A proper soil risk assessment needs to estimate the processes that affect the fate and the behaviour of a contaminant, which are influenced by soil biotic and abiotic components. For this reason, the measurement of biomarkers in soil bioindicator organisms, such as earthworms, has recently received increasing attention. In this study, the earthworm *Eisenia andrei* was used to assess the pollutant-induced stress syndrome after exposure to sublethal concentrations of Cd (10 or 100 μg g^{-1}) in OECD soil, after 14 d of exposure. Cadmium bioaccumulation and potential biomarkers such as catalase (CAT), hydrogen peroxide (H_2O_2), glutathione-S-transferase (GST), malondialdehyde (MDA), phenoloxidase (PO), metallothioneins (MTs) and genotoxic damage were determined. Results suggested that the exposure to 10 and 100 μg g^{-1} Cd significantly increased Cd bioaccumulation, MTs and MDA; 100 μg g^{-1} Cd contamination evidenced significantly higher values of H_2O_2 content and PO activity; CAT activity was inhibited at the higher concentration while GST and Comet assay did not show any significant differences from the control. Rank-based biomarker index showed that both different contaminated soils had an effect on the earthworms and allowed to validate the ecotoxicological relevance of this battery of biomarkers for a promising integrated multi-marker approach in soil monitoring and assessment.

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1. Introduction

Cadmium (Cd) compounds are largely produced by anthropogenic activities and widely spread in the environment (Lionetto et al., 2012). Similarly to other toxic heavy metals (e.g., Pb, Hg, Tl) and unlike harmful organic compounds, Cd accumulates in soil and its contamination may persist for many years as well as its effects on the edaphic fauna (Brusseau, 1997). For a proper evaluation of the eco-toxicological risk caused by Cd pollution, an estimation of the amount of contaminant crossing cell membranes, accumulating into an organism and affecting its biochemical processes should be assessed to support the data obtained by chemical analyses of the soil (i.e., Cd total concentration, speciation, potential bioavailability) (Simkiss et al., 2000).

Among the different edaphic species, earthworms can be used as biomonitors since they are always in close contact with the aqueous and solid phases of soil, and can accumulate pollutants in their body (Edwards, 2004). However, single biomarkers can give only a partial view of the health status of an organism exposed to a contaminant whereas an index-based indicator elaborated by

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integrating different biomarkers can provide a more robust tool for assessing the ecological risk of the contaminated environment under investigation (Gagné, 2014). Consequently, the application of a multi-biomarker approach in monitoring the effects of pollutants in ecosystems has been increasing in the last years (Calisi et al., 2013; Sforzini et al., 2015).

There exist many ways to integrate biomarkers such as the integrated biomarker response (Colacevich et al., 2011), the scale of classification method (Narbonne et al., 2005), rough set analysis of scaled biomarkers (Chèvre et al., 2003), and ranked-based biomarker index (Blaise et al., 2002). Among these, the rank-based approach is simple and has the advantage of working with nonparametric data distributions (Gagné, 2014). Blaise et al. (2002) illustrated how rank-based index could distinguish variations in biomarker responses in soft-shell clam (*Mya arenaria* Linnaeus) populations from heavy metals contaminated sites. Based on the previous considerations, the present work aimed at verifying for the first time the potential application of a rank-based biomarker approach on earthworms *Eisenia andrei* Bouché (Oligochaeta: Opisthopora: Lumbricidae) exposed to Cd contaminated artificial soils.

2. Materials and methods

2.1. Earthworms

Earthworms of *E. andrei* were taken from a breeding culture at the Department of Soil, Plant and Food Sciences, University of Bari Aldo Moro, Bari, Italy. They were reared on wet cow manure kept in a plastic container (dimensions: 35 × 45 × 60 cm) at room temperature in constant darkness. Sexually mature clitellate worms, about four months old and of 350–400 mg, were selected for each bioassay. They were rinsed with distilled water, laid on damp filter paper and stored in Petri dishes to void gut content within 24 h (in the dark at 22 ± 2 °C).

2.2. Experimental design

The artificial soil was prepared according to OECD guidelines (2004): 10% (dry weight) sphagnum, 20% kaolinite clay and 70% quartz sand; pH was adjusted to 6.1 ± 0.5 with $CaCO_3$. The test soils were artificially spiked with aqueous solutions of cadmium acetate to reach a final concentration of 10 and 100 μg of Cd per g (μg g^{-1}) of soil. Only distilled water was applied in the control. Soil was thoroughly mixed and moistened with distilled water up to 35% moisture content (by weight). The two concentrations of Cd were chosen to mimic the amount of Cd likely to be found in industrial contaminated soils (11.6–120 mg Cd kg^{-1}) (Marino and Morgan, 1999; Van Gestel et al., 2009) and were lower than the 14 d LC_{50} (374 mg Cd kg^{-1} dry weight) known for *Eisenia fetida* Savigny in OECD artificial soil (Fitzpatrick et al., 1996). Twelve earthworms per treatment were exposed for 14 d in 500 g (wet weight) of soil in three replicates and maintained at 20 ± 2 °C in constant light to ensure earthworms burrowing in the soil (Saint-Denis et al., 2001). At five-day intervals from the beginning of the exposure period, a small pellet (3 g dry weight) of uncontaminated cow manure was added to each replicate and soil moisture was restored by weight.

After the exposure period, earthworms were recovered, counted, rinsed in distilled water and left into Petri dishes for 24 h on wet filter paper to empty their bowel. Then, eight earthworms per replicate were frozen at −80 °C for metallothioneins (MTs) and Cd bioaccumulation analysis. The coelomic fluid (100 μl) of the remaining individuals per replicate was electrically extruded (Adamowicz, 2005) and pooled in eppendorf vials with 400 μl cold 0.1 M Phosphate Buffered Saline solution (PBS, pH 7.2). Few crystals of anticoagulant phenylthiourea (PTU) were added to a part of the pool that was used for the determination of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) content as well as catalase (CAT) and glutathione S-transferase (GST) activities. The remaining pool was not treated with any anticoagulant and was used for the determination of phenoloxidase (PO) activity and genotoxic damage (Comet assay). The total protein amount of each pool was assessed according to Lowry et al. (1951) (DC protein Assay Bio-Rad Kit) using Bovine Serum Albumin (BSA) as a quantitative standard. Where not specified, all chemicals were obtained by Sigma Aldrich.

2.3. Hydrogen peroxide (H_2O_2)

H_2O_2 content was assessed following the protocol proposed by Vašíček et al. (2011) using Amplex UltraRed Kit (Invitrogen, 2009). Two centrifugations (500 g, 5 min at 4 °C) separated coelomocytes from the coelomic fluid. Coelomocytes were counted on a Neubauer's chamber and adjusted to a final concentration of 1.0×10^6 cells/ml in 0.1 M PBS (pH 7.2). Cell suspension (100 μl) was mixed with 100 μl of working solution (50 μl of 10 mM Amplex UltraRed stock solution in dimethyl sulfoxide to 10 ml of 0.1 M PBS with 100 μl of horseradish peroxidase 10 U/ml) and incubated in the dark for 30 min. Amplex UltraRed reagent reacts with H_2O_2 in a 1:1 stoichiometric ratio to produce a red-fluorescent oxidation product (resorufin). The fluorescence of coelomocytes suspension was measured on a black microplate by means of a Perkin Elmer LS55 fluorimeter (excitation/emission wavelengths: 545/595 nm). The results were corrected by subtracting blanks (corresponding substrate in 0.1 M PBS, pH 7.2).

2.4. Catalase (CAT) activity

Total CAT activity was measured by assaying the hydrolysis of H_2O_2 according to Aebi (1984). Samples of coelomocyte suspension were thawed and 20 μl of them were mixed with 100 μl of 0.1 M PBS (pH 7.2) and 180 μl of Triton X 100 (1% v/v) to burst peroxisomes and release catalase. Enzyme reaction started with the addition of 100 μl of 30 mM H_2O_2 to 10 μl of the sample mixture and 190 μl of 0.1 M PBS.

Changes in absorbance were measured at 240 nm and the linear decrease in absorbance was recorded over 3 min by means of a microplate spectrophotometer (Epoch BioTek). Catalase activity was expressed in mmol of H_2O_2 reduced per min and mg of protein, using an extinction coefficient of 0.0394 mM^{-1} cm^{-1}. The results were corrected by subtracting blanks (corresponding substrate in 0.1 M PBS, pH 7.2).

2.5. Genotoxic damage (Comet assay)

The damage of DNA in a single coelomocyte was directly assessed by the Comet assay method (Fairbairn et al., 1995). Cells were stained with 0.4% Trypan blue and samples with at least 70% of cell viability were selected for the test. An amount of 75 μl for each selected suspension was mixed with Low Melting Point Agarose (stabilized to 37 °C in a water-bath). This mixture was poured on an agarose layer previously added and hardened on precoated slides. A further agarose layer was added on the slides before the treatment with cold lysis solution. Electrophoresis was then performed in an unwinding/electrophoresis solution for 30 min at a constant voltage of 0.74 Vcm^{-1}. The agarose micro-gel was stained with 300 μl of FLUOplus and incubated for 4 min. Finally, the slides were observed at 200× magnification under a Nikon Eclipse E400 fluorescent microscope equipped with FITC filters (excitation 450–490, dichroic minor 505, band pass 520). The images were recorded by a digital camera (Nikon Coolpix 995)

and analyzed by means of CometScore™ freeware software (TriTekCorp). The comets were screened recording the percentage of DNA in the tail as index of the DNA damage.

2.6. Glutathione S-Transferase (GST) activity

Total GST activity was assayed following the methods of Habig et al. (1974) and Ahmad and Pardini (1990). The samples were thawed and 40 μl were mixed with 70 μl of 20 mM glutathione (GSH in 0.1 M PBS, pH 7.2), 50 μl of 25 mM 1-chloro-2,4-dinitrobenzene (CDNB) (in 96% ethanol, 3:2 with water) and 440 μl of 0.1 M PBS(pH 7.2). The increase in absorbance of this mixture (GST activity), as consequence of the (CDNB)-glutathione conjugating reaction, was recorded at 340 nm for 3 min in a microplate spectrophotometer (Epoch BioTek) and expressed as nmol of GSH-CDNB conjugate formed per min and mg of protein using an extinction coefficient of 9.6 mM^{-1} cm^{-1}. The results were corrected by subtracting blanks (corresponding substrate in 0.1 M PBS, pH 7.2).

2.7. Malondialdehyde (MDA)

The produced MDA was determined using the thiobarbituric acid reactive substances (TBARS) method (Jain and Levine, 1995). The samples were thawed and 200 μl were mixed with 800 μl of 0.1 M PBS (pH 7.2) and 25 μl of butylated hydroxytoluene (0.04 M in ethanol) to prevent sample oxidation during the assay. Then, 500 μl of trichloracetic acid (30% w/v) were added, the mixture was cooled in ice for 2 h, and centrifuged at 2000 g for 15 min. One ml of each supernatant was mixed with 75 μl of 0.1 M ethylenediaminetetraacetic acid and 250 μl of thiobarbituric acid (1% w/v in 0.05 M sodium hydroxide). After incubation in boiling water bath for 45 min and cooling a room temperature, the absorbance of this mixture was measured at 532 nm by a microplate spectrophotometer (Epoch BioTek). TBARS concentrations were determined by using the extinction coefficient of the coloured complex (1.56×10^5 M^{-1} cm^{-1}) and expressed in nmol mg^{-1} protein. The results were corrected by subtracting blanks (corresponding substrate in 0.1 M PBS, pH 7.2).

2.8. Phenoloxidase (PO)

Total PO activity was measured using the procedure of Ashida and Soderhall (1984). Fresh sample of coelomic fluid (40 μl) was added to a microplate well containing 160 μl of 3,4-dihydroxy-DL-phenylalanine solution (3 mg ml^{-1} DL-DOPA in 0.1 M PBS, pH 7.2). The reaction was allowed to proceed for 30 min and the linear increase in absorbance at 492 nm was recorded with an Elisa reader (Labsystem Multiskan MS) every 5 min. Detection of PO activity in samples was carried out by measuring DL-DOPA transformation in dopachromes. The PO activity was expressed as absorbance units ($A_{492}min^{-1}$) per mg of proteins. The results were corrected by subtracting blanks (corresponding substrate in 0.1 M PBS, pH 7.2).

2.9. Metallothioneins (MTs)

The amount of MTs was analyzed on entire earthworms using the method of Gastaldi et al. (2007). Three frozen worms were homogenized in laboratory homogenizer (IKA) with 3 volumes of buffer (0.5 M sucrose in 20 mM Tris − HCl - pH 8.6, 6 μM leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 0.01% β-mercaptoethanol). The homogenated samples were centrifuged at 9000 rpm (NF 1200R centrifuge) for 30 min, at 4 °C. Then the supernatants were treated with ethanol/chloroform in order to obtain a partially purified MTs fraction by precipitation. MTs concentration in the samples was quantified indirectly by evaluating the sulphydrylic residue content. This latter was measured at 412 nm of absorbance (Pharmacia Biotech Ultrospec 4000 UV/Visible) using the Ellman's reagent (5,5′ −dithiobis-2-nitrobenzoic acid) and the GSH calibration curve. GSH contains one cysteine per molecule, thus it is a standard for quantifying sulphydryl groups in the sample. Data were expressed as nmol of sulphydrylic groups per g of wet weight of sample.

2.10. Cd bioaccumulation (CdB)

Five frozen earthworms from each replicate were thawed and dried in oven at 105 °C for 24 h. Dried worms were crushed in a mortar and 0.3 g were transferred into polytetrafluoroethylene tubes where 7 ml of HNO_3 (65%, TraceSELECT) and 1 ml of H_2O_2 (30%, TraceSELECT) were slowly added. The samples were let to react for one night at room temperature and then the mineralization was completed by using a microwave system (Multiwave 3000, Anton Paar). The dissolved samples were diluted to a final volume of 25 ml with distilled water, filtered through Whatman 42 filter papers, and analyzed for Cd concentration by ICP-OES (Inductively Coupled-Plasma-Optical Emission Spectroscopy) at 226.502 nm (Thermo iCAP 6000 series, Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.11. Statistical analysis

The analytical data were treated stastically using Statistica 9 StatSoft®. Statistical differences between treatments were determined through the non-parametric Mann−Whitney *U* test and the level of significance was set at $p < 0.05$.

Pearson's correlation test ($n = 9$, $p < 0.05$, two tailed) was performed to identify significant correlations between the variables.

2.12. Rank-based biomarker index

The biomarkers were scaled based on the mean value of each treatment which was ranked from 1 (lowest value) to 3 (highest value). If the difference between the control and the lower Cd content (10 μg g^{-1}) was not significant then the same rank number was attributed to each treatment. Conversely, if the difference was significant, then a higher rank value was attributed to the highest mean value. The process was then repeated between the lower (10 μg g^{-1}) and the higher (100 μg g^{-1}) Cd amount. The ranked values for each biomarker at each treatment were summed into an index (Gagné, 2014).

3. Results

All assayed earthworms were recovered alive at the end of the experiment and both Cd concentrations applied in the current trials were sub-lethal as expected.

The effects of these Cd concentrations on CAT, GST and PO activities of the coelomocytes are reported in Fig. 1. A significant ($p < 0.05$) decrease of CAT activity was detected for the treatment at 100 μg g^{-1} of Cd concentration in respect to the other treatments. A significant ($p < 0.05$) increase of PO activity was observed in samples treated with 100 μg g^{-1} of Cd only in respect to the control. No significant difference was observed between the control and the treatment at 10 μg g^{-1} of Cd concentration in soil for both CAT and PO activities. Vice versa, Cd did not exert any appreciable statistical pattern of influence on GST activity among all treatments and control. However, only PO activity displayed an increasing trend in relation to Cd concentration.

The H_2O_2 and MDA contents and the responses to the Comet

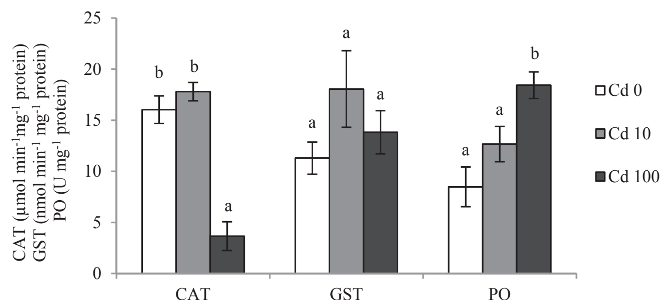

Fig. 1. Comparison of CAT, GST and PO enzymatic activities in coelomic fluid of *E. andrei* exposed to Cd in soil (µg g^{-1}). Means ± SE were reported. Different letters indicate a significant difference within the treatments, at the $p < 0.05$ level (non-parametric Mann–Whitney U test).

Table 1
H_2O_2, MDA and Comet assay biomarkers responses in *E. andrei* exposed to Cd in soil. Means ± SE are reported. Different letters indicate a significant difference within the treatments, at the $p < 0.05$ level (non-parametric Mann–Whitney U test).

Biomarkers	Cd soil (µg g^{-1})		
	0	10	100
H_2O_2 (Fl_{595} nm)	16.71 ± 0.86 a	26.1 ± 1.6 a	408 ± 55 b
MDA (nmol mg^{-1} protein)	1.67 ± 0.07 a	3.43 ± 0.42 b	2.60 ± 0.36 b
Comet (pixels)	10.3 ± 1.2 a	10.3 ± 1.0 a	7.97 ± 0.86 a

assay are reported in Table 1. The sublethal concentrations of Cd on coelomocytes of *E. andrei* exhibited a significant higher content of H_2O_2 only in earthworms exposed to 100 µg g^{-1} Cd, even though a weak increase was observed on earthworms exposed to 10 µg g^{-1} Cd in respect to the content detected in the control samples. Cadmium caused a significant ($p < 0.05$) increase of MDA content in both treatments at 10 and 100 µg g^{-1} of Cd contamination in respect to the control. No significant difference was observed between MDA contents in earthworms exposed to Cd at the two concentrations. The nuclei of the coelomocytes observed by fluorescent microscopy displayed a weak comet shape and no statistically significant difference in the percentage of DNA in the tail was detected among the treatments.

Significant differences on MTs (measured as sulphydrilic group content) and Cd bioaccumulation in the entire body of *E. andrei*

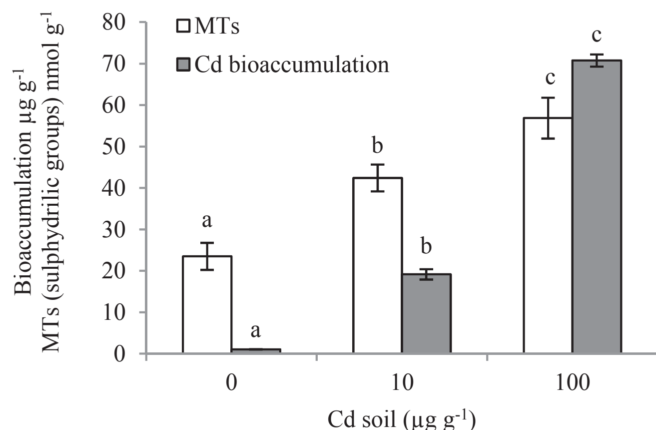

Fig. 2. Comparison of MTs content and Cd bioaccumulation among Cd treatments in the body of *E. andrei*. Means ± SE were reported. Different letters indicate a significant difference within the treatments, at the $p < 0.05$ level (non-parametric Mann–Whitney U test).

were observed in samples exposed to soil contaminated with 10 and 100 µg g^{-1} Cd compared also to the control (Fig. 2). An increase of MTs and Cd bioaccumulation was clearly observed in relation to increasing Cd concentrations.

Pearson's test identified significant correlations between some of the studied parameters (Table 2). Cd soil concentration (CdS), Cd bioaccumulation (CdB), H_2O_2, MTs and PO activities were positively correlated each to the other, whereas a negative correlation was observed between CAT activity and the above mentioned parameters. Finally, the GST activity was positively correlated with MDA and Comet assay was negatively correlated with PO activity.

The selected biomarkers have been integrated into the rank-based index and the corresponding indexes are reported in Table 3. Inversed score values were attributed to CAT activity because it was inhibited at the higher level of Cd contamination. According to the index, the control treatment was obviously the least impacted, with a sum of rank values of 7, and the 100 µg g^{-1} Cd treatment was the most affected one, with a sum of rank values almost double compared to the control. The sum of the indexes for the 10 µg g^{-1} Cd treatment was 9, slightly higher than that of the control.

4. Discussion

Earthworms accumulate metals efficiently into their body (Labrot et al., 1998; Stürzenbaum et al., 2004). The current trial confirms that *E. andrei* accumulated Cd in the body tissues at both Cd concentrations and according to a dose-dependent trend. The same trend was observed for MTs, whose amount was directly related to Cd concentration in soil and in the earthworms (Fig. 2, Table 3). MTs are inducible proteins involved in the homeostasis of essential metals and in protecting worms against xenobiotic heavy metals (House, 2009). Our data confirms previous studies (Ndayibagira et al., 2007) demonstrating a dose-responsive increase of MTs proteins in *E. andrei* exposed to increasing concentrations of Cd.

Usually, the exposure of living organisms to heavy metals leads to the production of reactive oxygen species (ROS), e.g. H_2O_2 (Valko et al., 2005; Dazy et al., 2009). CAT responds to the oxidative stresses by deactivating H_2O_2, thus preventing oxidative damages of biological macromolecules (lipids, proteins or DNA) (Valavanidis et al., 2006; Hyršl et al., 2007). Elevated ROS production was reported in response to tetrabromobisphenol A in *E. fetida* (Xue et al., 2009) and to Cu and Cd after *in vitro* exposure of *Eisenia hortensis* Michaelsen (Fuller-Espie et al., 2011). Also Pb can affect *E. andrei* inducing formation of ROS and inhibiting CAT activity (Saint-Denis et al., 2001). In our experiment, the highest H_2O_2 content and the lowest CAT activity were observed only at 100 µg of Cd per g of soil (Fig. 1, Table 1). Therefore, the inhibition of CAT activities is supposed at this higher Cd concentration whereas a hormetic phenomenon is presumed at the lower Cd concentration. A similar effect was documented in *E. fetida* exposed to Cd by contact with filter paper contaminated with Cd (Zhang et al., 2009a). Also Liu et al. (2010) demonstrated that the exposure of *E. fetida* to organic or inorganic pollutants induces stress responses of CAT activities which were basically promoted at low doses of the pollutants and inhibited at higher concentrations. Other studies reported that the CAT activity of *E. fetida* at a Cd contamination of 50 µg g^{-1} was significantly inhibited only after 28 d of exposure and not before that time. This observation is probably associated to the difficulty of the enzymatic system of the earthworms to fully compensate a long oxidative stress (Zhang et al., 2009b). However, an obvious strong and fast (after 14 h of exposure) inhibition of the expression level of CAT effectors was detected in *E. fetida* treated with 800 µg g^{-1} of Cd in soil (Brulle et al., 2006).

Table 2
Correlation analysis of Cd soil (CdS), biomarkers (CAT, H_2O_2, GST, MDA, PO, Comet, MTs) and Cd bioaccumulation (CdB).

	CdS	CAT	H_2O_2	GST	MDA	PO	Comet	MTs	CdB
CdS	1								
CAT	−0.94*	1							
H_2O_2	0.97*	−0.89*	1						
GST	−0.03	0.14	−0.14	1					
MDA	0.10	0.06	−0.04	0.84*	1				
PO	0.81*	−0.71*	0.80*	−0.10	0.21	1			
Comet	−0.60	0.65	−0.66	0.33	0.14	−0.76*	1		
MTs	0.81*	−0.75*	0.71*	0.35	0.47	0.72*	−0.40	1	
CdB	0.99*	−0.89*	0.95*	0.06	0.23	0.86*	−0.61	0.86*	1

*$p < 0.05$.

Table 3
Biomarker integration using the rank-based index of *E. andrei* exposed to Cd contaminated soil.

Biomarkers	Cd soil ($\mu g\ g^{-1}$)		
	0	10	100
CAT	1	1	2
H_2O_2	1	1	2
GST	1	1	1
MDA	1	2	2
PO	1	1	2
Comet	1	1	1
MTs	1	2	3
Sum of ranks	7	9	13

The exposure of earthworms to heavy metals can lead also to an increase of lipid peroxidation of cell membranes (Labrot et al., 1996; Saint-Denis et al., 2001). Lipid peroxides (LP) are unstable and decompose to form reactive carbonyl compounds among which MDA is the most abundant (Liu et al., 2009). Our results showed that MDA content was significantly higher in coelomocytes of *E. andrei* in both Cd treatments as compared to the control (Table 1). The sensitiveness to lipid peroxidation of this earthworm could be related to the abundance of polyunsaturated fatty acids (PUFAs) in their cell membranes (Albro et al., 1993). Our findings on Cd treatments are consistent with Xue et al. (2009) who showed that tetrabromobisphenol A induced the production of ROS in *E. fetida* and resulted in a significant increase of MDA content in the entire body.

PO activity was significantly higher in coelomocytes of earthworms exposed to 100 $\mu g\ g^{-1}$ Cd (Fig. 1). PO is a defense enzyme that mediates the melanization of infective pathogens and damaged tissues in invertebrates. Although there is a lack of studies on the heavy metal-specific influence of PO activity in earthworms, altered defense processes were observed in *Lumbricus terrestris* Linnaeus (Fugère et al., 1996) and *E. andrei* (Sauvé and Fournier, 2005) exposed to metal pollution.

GST is a ubiquitous enzyme that detoxifies many xenobiotics (Fournier et al., 1992). Zhang et al. (2009b) showed that GST activity in the post-mitochondrial fraction of *E. fetida* was significantly induced at 100 $\mu g\ g^{-1}$ of Cd. However, in our experiment no significant differences on GST activity in coelomocytes of earthworms were detected among the treatments (Fig. 1). This result is consistent with the non inducibility of GST reported by Stokke and Stenersen (1993), Borgeraas et al. (1996) and Honsi et al. (1999), even though La Course et al. (2009) showed that the highest GST activity occurred in the posterior part of the body and in the intestinal tissues of the earthworms.

Similarly, no significant difference of DNA damages among treatments was revealed by Comet assay (Table 1) in spite of the high sensitiveness of this assay: one break per chromosome or 200 breaks per cell (Siu et al., 2004). The Cd effect on the DNA tails of coelomocytes appeared to be not dose-dependent in the range of applied concentrations. Similar results (no DNA damage) were observed on *E. andrei* exposed for 28 d to an artificial soil spiked with 250 $\mu g\ g^{-1}$ Cd (Voua Otomo et al., 2014). These data are in contrast with Zhu et al. (2006) that observed a dose-dependent DNA damage in coelomocytes of *E. andrei* after 14 d exposure to soils spiked with 10 and 50 $\mu g\ g^{-1}$ of Cd.

Biomarkers of oxidative stress can be affected by several biological variables that contribute to the imbalance between oxyradical production and antioxidant defence (Colacevich et al., 2011). Thus, using multiple biomarkers and combining them into an integrated index of health status is a suitable approach for assessing sublethal effects in Cd-exposed earthworms. Herein, the rank-based index evidenced an increasing global stress as the Cd contamination in soil increased (Table 3). The index was only two scores higher than the control in samples contaminated with 10 $\mu g\ g^{-1}$ Cd, evidencing a slight toxicity towards earthworms, and six scores higher in the most contaminated samples (100 $\mu g\ g^{-1}$ Cd), suggesting a high toxicity. Also, the index increased as Cd concentration in the body increased. From our results and from data available in the literature (Brulle et al., 2006; Zhu et al., 2006), it is possible to summarize that the critical concentration of Cd sublethal toxicity for *E. andrei* after 14 d of exposure in contaminated soils is above 10 $\mu g\ g^{-1}$ Cd. The approach described in this paper could have some pitfalls, but it can be useful to identify polluted sites by using organisms collected from the field in which biomarker responses can be influenced by confounding environmental and biological factors (Marigómez et al., 2013).

Pearson's correlation analysis was performed to identify the strength of relationships between pair of variables. The results suggest that CdS, H_2O_2, CAT, PO, MTs and CdB are well correlated among them (Table 2). We can deduce that increased exposure to Cd in soil (CdS) involved the CdB ($r = 0.99$) and the production of H_2O_2 ($r = 0.97$) at 100 $\mu g\ g^{-1}$ Cd. This last could have inhibited the activity of the antioxidant enzyme CAT ($r = -0.89$) and the increase of the activity of the defensive enzyme PO ($r = 0.80$). Furthermore, Cd was detoxified upon binding to cysteine-rich proteins MTs ($r = 0.81$).

The presence of correlations between these variables is in agreement with the results of similar studies (Yuan et al., 2010; Chalkiadaki et al., 2014; Nannoni et al., 2014).

5. Conclusions

The rank-based index showed the discriminating effects of Cd pollution induced on a selected group of biomarkers of genetic, enzymatic and chemical type. At the concentration of 10 μg of Cd per g of soil, MTs and MDA increased their values; at the concentration of 100 $\mu g\ g^{-1}$Cd, discriminant biomarkers were CAT, H_2O_2, MDA, PO and MT. On the other hand, the Cd treatments did not

result in significant changes in GST activity and in Comet assay results. Among the selected biomarkers, MTs and MDA proved to be the most sensitive at 10 μg g^{-1}Cd, whereas H_2O_2 and CAT were the most efficient at 100 μg g^{-1}Cd. MTs seemed to be the most discriminating between the two applied doses. Finally, in our experiment, the biomarkers H_2O_2, CAT, PO and MTs resulted the most strictly correlated with CdS and CdB. These findings indicate that not all biomarkers respond to Cd contamination in the same way and confirm the importance of using a multi-biomarker approach to fully evaluate the health status of a bioindicator organism. Therefore, from a monitoring perspective, considering the complexity and costs of the proposed multi-biomarker approach, MTs, MDA, H_2O_2, and CAT can be a sufficient battery of biomarkers in ecotoxicological studies where earthworms serve as bioindicators of Cd pollution in natural soils.

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