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REVIEW

The role of earthworm defense mechanisms in ecotoxicity studies

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Abstract

Earthworms are important soil organisms that affect the soil structure by influencing organic and inorganic matter breakdown. Earthworms are in permanent contact with soil particles via their permeable skin and digestive tract and are thus strongly affected by pollutants present in the soil. Earthworms often live in very hostile environments with an abundant microflora and therefore have developed very potent defense mechanisms. These mechanisms have been described to be influenced by various types of organic and inorganic pollutants and also by the nanoparticles that reach the soil system. Reduced abilities of earthworms to protect themselves against pathogenic microorganisms result in lower reproduction rates and increased mortality. In this review, a summary of the up-to-date data describing the effects of contaminants on the natural defense barriers and immune system of earthworms is presented.

Key Words: pollution; immune system; earthworms; biomarker

Introduction

Earthworms (Lumbricidae, Annelida) are protostomian organisms with a true celom that is filled with celomic fluid containing free celomocytes. The celomic cavity is metameric, and the segments are separated by transversal septa. Each segment of the cavity interfaces with the outer environment via a pair of metanephridia and a dorsal pore that enables microorganisms to enter the celomic cavity. Therefore, the celomic fluid is not aseptic and contains bacteria, fungi and protozoa from the outer environment. The growth of these microorganisms is kept under control by various cellular and humoral innate defense mechanisms that will be described in detail in the following section.

Earthworms are the most abundant invertebrates in the soils of temperate regions and are extremely important for soil formation (Edwards, 2004). Earthworms participate in nutrient cycling in terrestrial ecosystems and in the formation of the soil profile from the physical, chemical and microbial perspectives (Bartlett *et al.*, 2010). They improve its structure by increasing the macroporosity, which affects aeration, water dynamics and organic and inorganic matter breakdown (Wen *et al.*, 2006; Ruiz *et al.*, 2011). Earthworms are permanently in close contact with soil particles and microorganisms present in the soil via both a highly permeable skin and an alimentary tract (Jager *et al.*, 2003; Drake and Horn, 2007). Therefore, they are significantly affected by the pollutants that reach the soil system and are thus well suited for the monitoring of soil contamination. Different earthworm species have different effects on soil formation because of their different behavioral patterns. Epigeic earthworms live above the mineral soil, rarely form burrows and preferentially feed on plant litter. Endogeic species live below the surface, where they build predominantly horizontal burrows. These species ingest large amounts of mineral soils and humified material. Anecic earthworms build permanent vertical burrows deep into the mineral soil layer and come to the surface to feed on decomposed plant litter and other organic residues (Lee, 1985). Two epigeic species, *i.e.*, *Eisenia fetida* and *Eisenia andrei*, have been used for many years to monitor ecotoxicity. There are two sets of guidelines, *i.e.*, those from the Organization for Economic Co-operation and Development (OECD) and those from the International Organization for Standardization (ISO), for the assessment of the ecological risk of contaminated soil, the determination of the acute toxicity of chemicals on earthworms (OECD, 1984;

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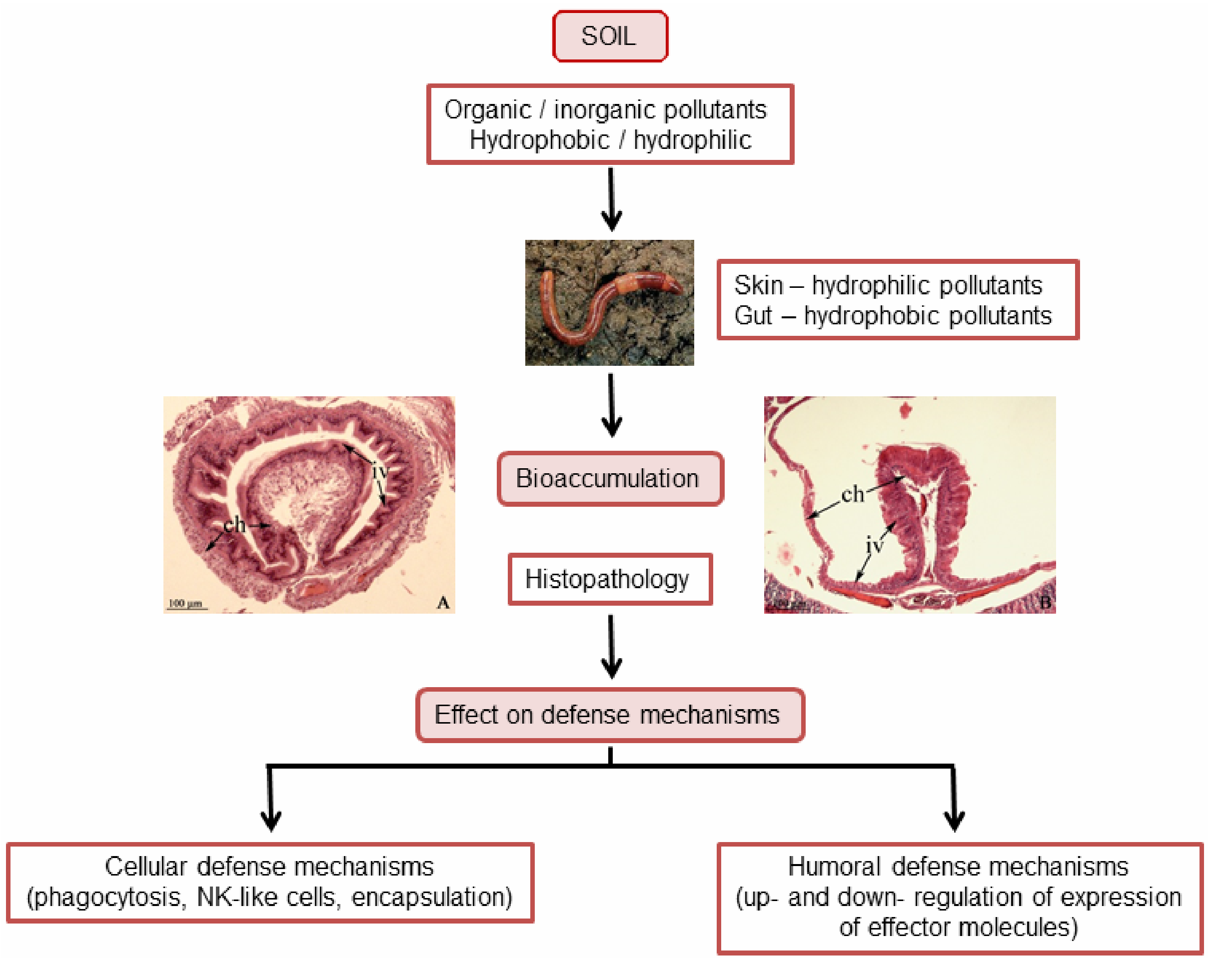

Fig. 1 Earthworms are affected by the presence of pollutants in the soil. Hydrophilic contaminants enter the earthworm body predominantly through the skin, whereas hydrophobic substances enter via the digestive tract. Pollutants are accumulated in earthworm tissues, which can result in tissue and cell disruption, such as progressive reduction of intestinal villi (iv) and chloragogenous tissue (ch) in earthworms kept in dioxin-polluted soil (B; Roubalova *et al.*, 2014). Additionally, both cellular and humoral defense mechanisms are impaired by the soil contaminants.

ISO, 1993), and the effect on their reproduction (ISO, 1998; OECD, 2004).

Earthworms have been described to bioaccumulate contaminants, such as various organic pollutants (Jager *et al.*, 2005), heavy metals (Nahmani *et al.*, 2007) and nanoparticles (Canesi and Prochazkova, 2014). They are able to take up chemicals from pore water through their skin and via soil ingestion. According to the model developed by Belfroid *et al.* (1995), the ingestion of sediment can be the dominant uptake route of hydrophobic compounds with logK_{ow} values > 5. The presence of contaminants in the soil disturbs major physiological functions of earthworms, such as survival, nutrition, immunity, growth, and reproduction, and these effects depend on the matrix, exposure time, and the types and doses of the pollutants in the environment. In recent years, there has been a growing interest in increasing our knowledge of the biological responses of earthworms to pollutants in order to standardize a suite of biomarkers of the responses to soil chemical pollution (Beliaeff and Burgeot, 2002). Biomarkers detect the effects of contamination at an early stage before sublethal effects, such as inhibition of growth and reproduction, become apparent. The biomarker approach represents a very useful tool in monitoring stress response to pollutants in field populations (Kammenga *et al.*, 2000; Hankard *et al.*, 2004). The choice of appropriate biomarkers is crucial for monitoring the effects of pollution on organisms. Reactions to pollution may be monitored on various levels, the whole body level (viability, weight loss, reduction of reproduction, and escape reaction), the organ and tissue level (histopathological changes), the cellular level (decrease in the physiological conditions of the cells) and the molecular level (the up- and down-regulation of the expression levels of

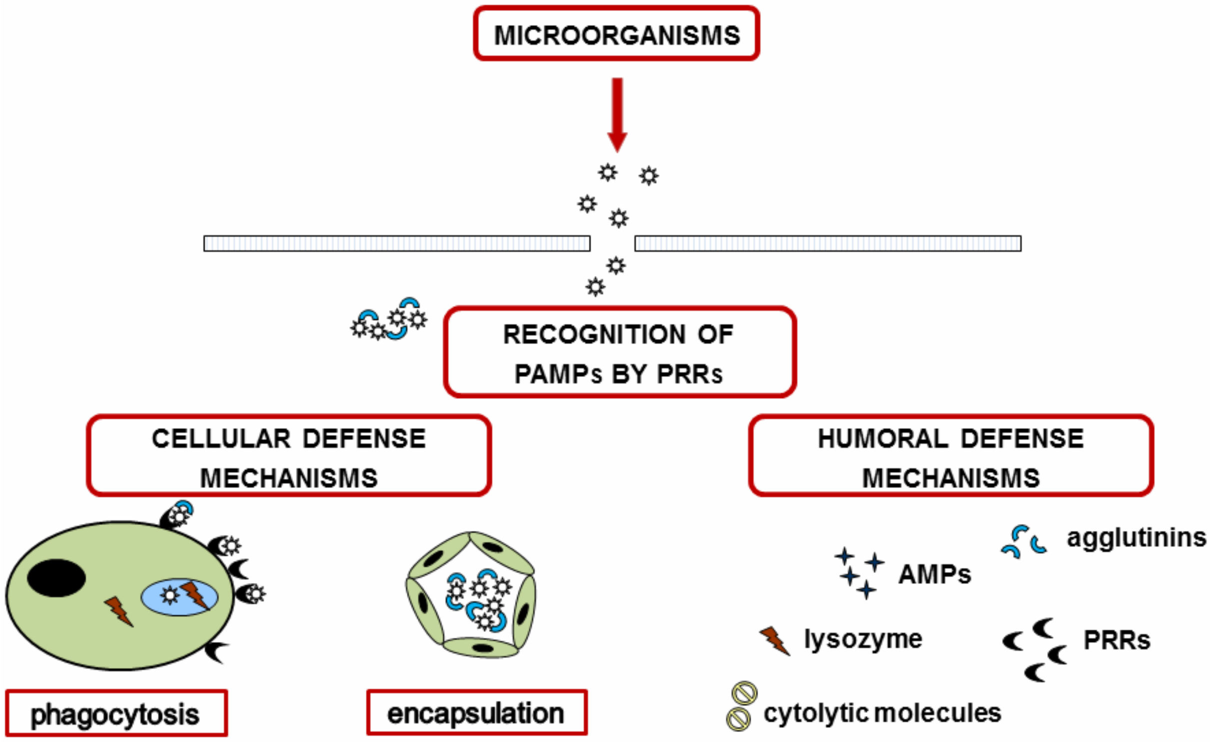

Fig. 2 The general scheme of the innate defense mechanisms in earthworms. The first protective barrier of earthworms is the skin in combination with the secreted mucus that contains various antimicrobial factors. Invading microorganisms are recognized by both soluble and membrane-bound pattern recognition receptors (PRRs) that sense pathogen-associated molecular patterns (PAMPs). On the basis of this recognition, microorganisms are phagocytized by coelomocytes or agglutinated and subsequently encapsulated. Moreover, genes encoding various humoral factors involved in the elimination of invaders are expressed, such as antimicrobial peptides (AMPs), cytolytic molecules, agglutinins, lysozyme and various soluble PRRs that trigger the activation of the prophenoloxidase cascade.

genes that are sensitive to the environmental changes, transcriptome profiling) (Owen *et al.*, 2008; Asensio *et al.*, 2013; Calisi *et al.*, 2013; Roubalova *et al.*, 2014; Sanchez-Hernandez *et al.*, 2014; Sforzini *et al.*, 2015) (Fig. 1). Although these responses may indicate the disturbances at the level of populations, only few data link biomarker level with effects on the functioning of earthworms in ecosystems (Maboeta *et al.*, 2003; Spurgeon *et al.*, 2005; Plytycz *et al.*, 2009).

The effects of pollutants on the defense mechanisms of earthworms

Similarly to other invertebrates, earthworms rely on natural nonspecific innate immunity for defense and lack anticipatory, specific and lymphocyte-based immune mechanisms. Additionally, the natural barriers of earthworms represent the first line of protection against the invasion of microorganisms. A brief summary of earthworm immune mechanisms is shown in Figure 2. In the following sections, the effects of various soil pollutants on the nonspecific defense barriers and the cellular and humoral mechanisms of immunity are reviewed.

Natural defense mechanisms and pollution

The first nonspecific protective barrier in earthworms is the skin, which consists of the epidermis and a thin cuticle that covers the entire body. The epidermis is formed by a single-layer epithelium of supporting cells, basal cells that have an important role in wound healing and graft rejection, and secretory cells that secrete mucus containing mucopolysaccharide-lipid-protein complex (Alves *et al.*, 1984; Bernaldo de Quiros and Benito, 1986) that serves as a lubricant during locomotion and contains several antimicrobial factors (Valembois *et al.*, 1986). The cuticle contains mucopolysaccharides that act as an antimicrobial barrier (Rahemtulla and Lovtrup, 1974).

Both cuticle and mucus production can be affected by the inorganic and organic contaminants as well as nanoparticles present in the soil. The exposure of the earthworms *Lumbricus rubellus* and *Lumbricus variegatus* to C60 fullerene nanoparticles has been described to result in cuticle damage with underlying pathologies of the epidermis and muscles (Pakarinen *et al.*, 2011; Van Der Ploeg *et al.*, 2013). Furthermore, the exposure of *E. fetida* to sub-lethal concentrations of 1,2,4-trichlorobenzene

Table 1 Summary of recent studies involving genotoxicity assessment of various organic and inorganic pollutants

Tested species	Organic pollutant	Source	Reference
E. fetida	naphtenic acids	constituents of petroleum, used in commercial and industrial applications	(Wang *et al.*, 2015a)
E. fetida	di-n-butyl phthalates	increase the plasticity of many materials	(Du *et al.*, 2015)
E. fetida	benzo[a]pyrene	the result of incomplete combustion	(Duan *et al.*, 2015)
E. fetida	triclosan	antimicrobial additive used in personal care products	(Lin *et al.*, 2014)
E. fetida	metalaxy-M	fungicide	(Liu *et al*. 2014)
E. fetida	azoxystrobin	fungicide	(Han *et al.*, 2014)
E. fetida	chlortetracycline	veterinary antibiotics	(Lin *et al.*, 2012)
E. andrei	B[a]P, TCDD	by-products from a number of human activities	(Sforzini *et al.*, 2012)
E. fetida	toluene, ethylbenzene and xylene	associated with crude petroleum and petroleum products	(Liu *et al.*, 2010)
Tested species	**Inorganic pollutant**	**Source**	**Reference**
E. andrei	Cd, Zn	metals provided in the form of $CdSO_4$, $ZnSO_4$	(Otomo *et al.*, 2014)
E. fetida	Cr, Cu, Ni, Pb, Zn	soils subjected to chemical characterization and total main heavy metal quantification	(Zheng *et al.*, 2013)
L. castaneous, *D. rubidus*	As	concentrations of arsenic elevated due to mining	(Button *et al.*, 2012)
A. caliginosa, *E. fetida*	Cu, Cd	sites near roads with heavy traffic	(Klobucar *et al.*, 2011)
E. andrei	Be, Al, Ba, Mn, Fe, Ni, Zn, U	deposition of mine tailings and sludge, runoffs from the aquatic system	(Lourenco *et al*. 2011)
E. fetida	Ni, Cr(III), Cr(VI)	pollutants used in numerous industrial processes	(Bigorgne *et al.*, 2010)
E. fetida	Cd, Pb	toxic elements widely distributed in the environment	(Li *et al.*, 2009)

results in ultrastructure alterations of the cuticle and skin, and the reduction of mucus production by secretory cells. At higher concentrations, mucus production disappears, and the cuticle is loosened and weakened (Wu *et al.*, 2012a). Exposure of the earthworm *E. fetida* to soil containing tetraethyl lead (TEL) and lead oxide (a gasoline additive) causes ruptures of the cuticle and skin, extrusion of the coelomic fluid and inflexible metameric segmentation (Venkateswara Rao *et al.*, 2003).

Cellular innate immunity

The celomic fluid of earthworms contains different types of cells that are generally termed celomocytes. The nomenclature of celomocytes is based on differential staining, ultrastructure, and granular composition. There are two basic categories of celomocytes. Amebocytes function primarily in immune reactions, such as phagocytosis, encapsulation, nodulation as well as humoral immune responses, and mainly nutritive eleocytes (Sima, 1994). Celomocytes have been described to respond to a wide range of pollutants and therefore are often used in soil ecotoxicology assessment.

At the cellular level, two immune system-related parameters have been used as sensitive sub-lethal endpoints in assessment of the toxicity of pollutants in earthworms: phagocytosis and NK-like cell activity. Phagocytosis represents an important defense mechanism that begins with the recognition of non-self, which is followed by the engulfment and destruction of phagocytosed particles. Engulfed material can be eliminated by proteolytic and lysosomal enzymes or by an oxidative burst that involves the production of highly reactive oxygen radicals. The inhibition of phagocytosis in earthworms that are exposed to various metals and organic substances, such as polychlorinated biphenyls (PCBs) and polychlorinated dibenzo-p-dioxins/dibenzofurans (PCDDs/Fs), has been described (Ville *et al.*, 1995; Fugere *et al.*, 1996; Fournier *et al.*, 2000; Sauve *et al.*, 2002; Belmeskine *et al.*, 2012). Silver nanoparticles have been shown to be accumulated predominantly in the amebocyte population of celomocytes with subsequent selective cytotoxicity of these cells (Hayashi *et al.* 2012). Furthermore, some celomocytes have been shown to possess cytotoxic activity similar to that of natural killer (NK) cells. These cells exhibit rapid response to allogenic structures and have been described to be involved in the rejection of allografts (Suzuki and Cooper, 1995). The NK-like cell activity has been demonstrated to be suppressed by polyaromatic hydrophobic hydrocarbons (PAHs) (Patel *et al.*, 2007), PCBs (Suzuki *et al.*, 1995), and PCDDs/Fs (Belmeskine *et al.*, 2012). Furthermore, flow cytometry has revealed a lower frequency of immune cells (amebocytes) in contrast with metabolic eleocytes in earthworms that have been exposed to metal- and radionuclides-contaminated soil (Lourenco *et al.*, 2011).

At the subcellular level, the lysosomal membrane stability system has been identified as a specific target of the toxic effects of contaminants (Moore, 1990). Lysosomal membrane integrity can be measured with the neutral red retention assay (Weeks and Svendsen, 1996). The stability of the membranes has been shown to decrease with increasing stress due to the presence of pollutants in the environment (Moore, 1985; Booth and O'Halloran, 2001; Booth *et al.*, 2003).

Because many soil contaminants exert genotoxic activities that result in DNA damage in the celomocytes, it is used as an important tool in environmental biomonitoring. The most widely used genotoxocity biomarker is the comet assay; this method has been shown to be appropriate for measuring DNA damage in the individual cells of both vertebrates and invertebrates (Singh *et al.*, 1988; Fairbairn *et al.*, 1995; Cotelle and Ferard, 1999; Faust *et al.*, 2004; Sforzini *et al.*, 2012). In Table 1, examples of organic and inorganic pollutants described in recent studies that cause DNA damage are listed.

Humoral defense mechanisms

Molecules involved in innate immunity

The celomic fluid of annelids exhibits numerous biological activities that are involved in the defense mechanisms against invaders (Fig. 2). The recognition of microbial pathogens is mediated by pattern recognition receptors (PRRs) that sense so-called pathogen-associated molecular patterns (PAMPs). These structures are common among microorganisms and include, *i.e.*, the lipopolysaccharides of Gram-negative bacteria, constituents of the peptidoglycan of Gram-positive bacteria, β-glucans of yeasts and viral double-stranded RNA. This recognition results in the activation of both cellular and humoral defense mechanisms, including the production of antimicrobial proteins and peptides (Joskova *et al.*, 2009), and the activation of an important invertebrate defense mechanism termed the prophenoloxidase cascade (Beschin *et al.*, 1998; Soderhall and Cerenius, 1998).

To date, only two PRRs in earthworms have been described, *i.e.,* celomic cytolytic factor (CCF) (Beschin et al., 1998; Bilej *et al.*, 1998, 2001) and Toll-like receptor (TLR) (Skanta *et al.*, 2013), and these PRRs recognize various PAMPs. The expression of CCF has been described to be significantly down-regulated in *L. rubellus* following lifelong exposure to C60 nanoparticles, which suggests the induction of immunosuppression (Van Der Ploeg *et al.*, 2013). Dioxins have also been shown to affect the expression of CCF (Roubalova *et al.*, 2014).

A wide range of antimicrobial molecules that are involved in killing the microorganisms that enter the earthworms´ bodies have been described. Celomic fluid has been documented to contain various antimicrobial factors, such as lysozyme (Çotuk and Dales, 1984; Joskova *et al.*, 2009) and antimicrobial peptides (Wang *et al.*, 2003; Liu *et al.*, 2004; Li *et al.*, 2011). Among the factors that are involved in humoral immunity, particular interest has been devoted to the cytolytic components that are secreted by celomocytes. The cytolytic activity of the celomic fluid was originally demonstrated on vertebrate erythrocytes and the resulting effect was described as hemolysis. The majority of identified hemolysins exhibit broad spectra of antibacterial and/or bacteriostatic activities against pathogenic soil bacteria (Roch *et al.*, 1991; Milochau *et al.*, 1997; Eue *et al.*, 1998). Various types of pollutants, such as metallic compounds (Brulle *et al.*, 2008; Mo *et al.*, 2012) and TiO_2 nanoparticles (Bigorgne *et al.*, 2012), have been described to influence the expression of these molecules and therefore cause inappropriate immune response to invading pathogens. Earthworm calreticulin is a highly conserved calcium-binding protein that has also been shown to be affected by the presence of various pollutants in soils (Chen *et al.*, 2011; Roubalova *et al.*, 2014). It participates in the regulation of Ca^{2+} homeostasis, acts as a chaperone and is involved in the regulation of cell signaling (Wang *et al.*, 2012). It also plays a role in the stress response and immune reactions (Goo *et*

Table 2 List of pollutants that affect the activity and gene transcription of antioxidant enzymes

Pollutants that affect activities of antioxidant enzymes			
Tested species	**Type of pollutant**	**Enzymes affected by pollutants**	**Reference**
E. fetida	decabromodiphenyl ether	SOD, CAT, POD	(Zhang *et al*., 2014)
E. fetida	phenanthrene	SOD, CAT, POD	(Shi *et al*., 2013)
E. fetida	multi-metal-contaminated soil (Cd, Cr, Cu, Ni, Pb, and Zn)	SOD	(Zheng *et al*., 2013)
E. fetida	phenanthrene, pyrene	SOD, CAT	(Wu *et al*., 2012b)
E. fetida	chlortetracycline	SOD, CAT	(Lin *et al*., 2012)
E. fetida	ZnO nanoparticles	SOD	(Li *et al*., 2011)
Pollutants that affect gene expression of antioxidant enzymes			
Tested species	**Type of pollutant**	**Genes affected by pollutants**	**Reference**
E. fetida	naphthenic acids	SOD, CAT	(Wang *et al*., 2015b)
E. fetida	2,2',4,4'-tetrabromodiphenyl ether	SOD, CAT	(Xu *et al*., 2015)
E. fetida	copper sulphate ($CuSO_4$)	SOD, CAT	(Xiong *et al*., 2014)
E. fetida	silver nanoparticles	SOD, CAT	(Hayashi *et al*., 2013)
E. fetida	zinc oxide (ZnO)	SOD, CAT	(Xiong *et al*., 2012)
E. fetida	galaxolide, tonalide	SOD, CAT	(Chen *et al*., 2011)

al., 2005; Kuraishi *et al*., 2007; Silerova *et al*., 2007; Gold *et al*., 2010).

Enzymes involved in oxidative stress

Aerobic organisms developed efficient antioxidant defense system to protect themselves against reactive oxygen species (ROS). The major source of intracellular ROS is the mitochondrial respiratory chain (Han *et al*., 2001; Ott *et al*., 2007), and these radicals are also produced in smaller amounts in other cell compartments, such as the endoplasmic reticulum, the plasma and nuclear membranes, and by some oxidases (Mittler *et al*., 2004; del Rio *et al*., 2006; Navrot *et al*., 2007). Free radicals were described to have an important role in cell signaling (Mates *et al*., 2002; Scandalios, 2005; Mates *et al*., 2008) and protection against invading pathogens (Babior *et al*., 1975; Rossi *et al*., 1985; Nacarelli and Fuller-Espie, 2011). Oxidative stress induces DNA modifications (Bohr, 2002), direct oxidation and inactivation of iron-sulfur (Fe-S) proteins (Fridovich, 1997), lipid peroxidation (Arai, 2014), and apoptotic events by means of caspase dependent pathways (Bearoff and Fuller-Espie, 2011). Under stressful conditions (*e.g.,* exposures to UV radiation, organic and inorganic contaminants, extreme temperatures and biotic stress), the concentrations of ROS increase, resulting in the development of oxidative stress and subsequent damage to cellular structures (Foyer and Noctor, 2005; Gill and Tuteja, 2010; Tumminello and Fuller-Espie, 2013). Antioxidant enzymes are considered to be a primary defense that protects biological macromolecules from oxidative damage. Three groups of these enzymes play significant roles in protecting cells from oxidant stress, *i.e*., superoxide dismutases (SODs), catalase (CAT) and peroxidases (PODs) (Mates, 2000). SODs are a ubiquitous family of metal-containing enzymes that depend on bound manganese (mitochondrial SOD), copper or zinc (intra- and extra-cellular SODs) for their antioxidant activity. SODs efficiently catalyze the dismutation of superoxide anions into hydrogen peroxide, which is substantially less toxic than superoxide, and oxygen. CAT and PODs degrade hydrogen peroxide to water. Both the enzyme activities and gene expression levels of antioxidant enzymes are frequently used to determine the effects of pollution on earthworms (Table 2).

Conclusions

This review summarized the data that have been published so far regarding the effects of various soil pollutants on the defense mechanisms of earthworms. The toxicities of these chemicals, which often enter the food chain, have been described to affect the immune system of not only invertebrates but also vertebrates, including humans. This article illustrated the various mechanisms through which the effects of pollutants are mediated on both the cellular and humoral components of the immune system. Such disruptions of the abilities of earthworms to protect themselves against invading pathogens has been shown to be closely related to the reduced reproduction and growth rates of earthworms and increased mortality. Moreover, these immunological parameters can be used as reliable biomarkers for the detection of the pollutant-induced responses of soil organisms.

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