USE OF LUMBRICIDES SPECIES AS BIOLOGICAL INDICATORS OF ENVIRONMENTAL POLLUTION WITH METALODISRUPTERS

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Abstract

Based upon the ecological and trophic features of lumbricides, it is demonstrated in studies performed worldwide that these animal species may represent preferential organisms for the process of bioaccumulation of heavy metals. Environmental pollutants such as cadmium (Cd), lead (Pb), manganese (Mn), cobalt (Co), nickel (Ni), copper (Co), zinc (Zn), selenium (Se) and mercury (Hg) were identified in the body of lumbricides using sensitive methods as for example atomic absorption spectrometry (AAS) and inductively coupled plasma mass spectroscopy (ICP-MS). Several tisular biomarkers of exposure to heavy metals have been characterized in lumbricides. Of these, methoxyrheorufin-O-deethylase (MROD), NADH and NADPH cytochrome-reductases, glutathione-S-transferase (GST), cytochrome P-450, catalase (CAT), glutatione-reductase (GR), acethylcholinesterase (AchE), lipide peroxidase (LP), the total glutathione concentration (GSH) and the percent of oxidizied gluthatione (% GSSG) deserve to be mentioned. These biomarkers are sensitive to exposure to metalodisrupters. These observations, evidenced in the majority of lumbricide species studied, may justify the use of lumbricides in environmental quality monitoring with respect to heavy metals pollution.

Key words: earthworm, soil, pollutant, enzyme, endocrine disruptors

There is evidence that heavy metals may act as disruptors for the endocrine system, leading to endocrine disorders, in particular to reproductive endocrinology pathology. Mainly, heavy metals interfere with the endocrine system by acting as agonists or antagonists at the endocrine receptor level.

Steroid hormones receptors are part of the family of nuclear receptors. The hormone-nuclear receptor complex binds to hormone response elements located in the DNA chain of the target gene, to subsequently activate DNA transcription. The DNA binding domain is highly specific: two zinc (Zn) fingers, coordinated by 4 cysteine residues were identified to be involved in the process of receptor binding. It is demonstrated that the Zn atom from the Zn binding sites of the estrogen receptor can be replaced by several metals such as copper (Co), cobalt (Co), nickel (Ni) and cadmium (Cd). Substitution of Zn by Ni or Cu results in inhibition of the estrogen receptor to DNA. Several data indicated that even exposure to low Cd concentrations interferes with the physiological effects of sex hormones in both males and females. First, Cd disrupts steroidogenesis, including the biosynthesis of androgens, progesterone and estrogens, thus resulting in hypogonadism and sex differentiation disorders [21]. Second, Cd exhibits both estrogen- and androgen-*like* properties *in vivo* and *in vitro*, by direct binding to estrogen and androgen receptors, respectively. *In utero*, exposure to Cd profoundly interferes with the intrauterine growth of the fetus [21]. Recently, a lithuanian study has evidenced significantly higher Cd levels in breast tissue and biological media in women with breast cancer compared to controls, thus, suggesting that exposure to Cd could be interpreted as a potential risk factor for breast cancer [17].

Another heavy metal, arsen (As) binds to the glucocorticoid receptor and interferes with the activity of glucocorticoid hormones. In contrast to pesticides, acting as estrogen receptor-agonists, As acts as an hormone antagonist.

Mices exposed to Co develop testicular atrophy and degenerescence. A study in which mices from the B6C3F1 line were exposed to increased concentrations of Co sulphate in the air, showed atrophy of the testes in males and increased length of the estrous cycle in females, respectively. Additionally, experimental exposure to Co resulted in low motility of spermatozoa, teratospermia and subnormal epididimal and testicular weight.

It is indicated by the literature that both organic and anorganic forms of mercury (Hg) show important bioaccumulation in the liver, the kidney, and also in many of the major endocrine glands (*i.e.* the hypothalamus, the pituitary, the thyroid, the testes, the ovaries, the adrenal cortex and the uterus) in laboratory animals, wild and even human. Alterations of steroidogenesis by Hg leads to diminished levels of sex hormones thus affecting sexual differentiation and reproduction; moreover, Hg appears to interfere with the hypothalamo-pituitary-thyroid gland activity, all together suggesting its role as a major endocrine disruptor [20].

As most of heavy metals are soil pollutants, it is needed to identify soil species behaving as biological indicators of the presence of xenobiotics and the degree of exposure. Among these species, a key role is played by lumbricides, whose behavior as biological indicator in polluted soils can be evaluated by several tests.

Earthworms are common components of the soil invertebrates population and are widely used as bioindicators of soil quality. Earthworms are important components of the soil system, mainly because of their favorable effects on soil structure and function. Their burrowing and feeding activities contribute notably to increased water infiltration, soil aeration, and the stabilization of soil aggregates. In addition, earthworms help to increase soil fertility by formation of an organic matter layer in topsoil [4]. These features, among others, have led to the popularity of earthworms as excellent biological indicators of soil pollution. These organisms ingest large amounts of soil, or specific fractions of soil (*i.e.,* organic matter), thereby being continuously exposed to contaminants through their alimentary surfaces. Moreover, several studies have shown that earthworm skin is a significant route of contaminant uptake as well [14].

Tests on earthworms aim to evaluate their response at the individual or population level (community census analysis, nematode maturity index), to assess the effects on worms reproduction, to determine cellular and subcellular functions (lisosomal stability), to evaluate the activities of enzymatic systems or the function of the immune system, to establish heat-shock protein induction and to analyse DNA alterations or the expression of specific genes (annetocin) [12].

The worm reproduction test evaluates the impact of soil pollutants on sublethals parameters in earthworms. The primary objective of the test is represented by effects on diet, weight and the reproduction rate in adults earthworms. This last parameter can be expressed as the number of cocoons/adults/ week, the number of newborn/adults/week and the number and weight of live newborn/cocoon. The test can be applied to evaluate contamination of soil with heavy metals and pesticides.

Exposure to Cu and Zn of the species *Eisenia foetida* resulted in a reduced number of juveniles, as shown by [9]. Prolonged contamination of soil with Pb, Cd and Zn resulted in a low production and low viability of cocoons at species *Eisenia foetida, Eudrilus eugeniae* and *Perionyx excavatus,* as observed in studies. It is recommended that this test should be used as an sensitive indicator test of pollution. When exposed to Zn, the species *E. foetida*, *L. rubellus*, *L. terestris*, *Aporrectodea caliginosa* presented a diminished survival rate, reduced body weight and reduced rate of cocoon production [15]. In a study performed in Poland, it was shown that Cd inhibits spermatogenesis in *Dendrobaena veneta* [16].

The lysosomal membrane stability test evaluates the lysosomal membrane fragility caused by exposure to pollutants. Destabilization of the lysosomal membrane is a nonspecific phenomenon, that appears as a response to organic and anorganic toxic compounds and correlates with the degree of stress induced by the contaminant. More recently, a new method to assess the permeability of the lysosomal membrane was developed – *the neutral red retention time* (NRRT). The method is based on the fact that neutral red is retained only by healthy lysosomes. Therefore, the NRRT represents a marker of the lysosomal membrane permeability. In earthworms, an inverse dose-response relationship of the NRRT was initially demonstrated for *Lumbricus rubellus* and *Eisenia andrei,* with significant decreases in NRRT after exposure to sublethal Cu concentrations.

Both the NRRT and life cycle parameters were studied in 4 worm species (*Eisenia fetida, Lumbricus terrestris, Lumbricus rubellus* and *Aporrectodea caliginosa)* exposed to Zn by Spurgeon et al. [16]. In all cases, NRRT represented the most sensitive parameter. Spurgeon et al. [16] demonstrated that exposure to Cd, Pb, Mn, Co, Ni, Cu and Zn of *Lumbricus terrestris* results in a reduced NRRT. In the species *Eisenia Andrei*, the stability of the lysosomal membrane in the coelomocytes can be used as a biomarker of pollution with Cu, as shown by [3].

Immunologic tests evaluate alterations of the immune system associated to contamination with pollutants. The immune system is deeply involved in response of the body to infection. Various effects are generated by the interactions between xenobiotics and the immune system, that is these compounds may either stimulate or suppress immune functions. Humoral immunity in earthworms is supported by aglutinins and lytic factors produced by chloragocytes, a subpopulation of phagocytic coelomocytes in earthworms. Humoral immunity was assessed by factors such as lysosym activity, proteases, hemolysis and antibacterian activity. An efficient response of the biomarkers was obtained after exposure to several heavy metals compounds, *i.e.* Cu, Cd, Hg, Pb and Zn. The most frequently used screening parameters of imunologic effects of metals are determination of the phagocytic activity and formation of the unistratified rosette by adhesion of xenobiotics to coelomocytes.

A study performed in Great Britain on the species *Lumbricus terrestris* shows changes in immunologic tests after exposure to heavy metals such as Pb, Mn, Co, Ni, Cd and Zn [16]. Lysosomal storage of lypofucsin in the chloragocytic tissue and that of neutral lipids in the coelomocytes of *Eisenia Andrei* may be used as biomarkers of exposure to Cu [3] while quantifying lipids peroxides (LP) and peroxidizable lipids in the same species may indicate soil contamination with Pb [13]. Exposure to Zn, Pb, Cu and Cd significantly affects the activity and viability of coelomocytes (the immune function) in *Allolobophora chlorotica* [5].

Given the overall sensitivity of the MFO system to several pollutants, the *analysis of the concentration and activity of enzymes that are components of the mixed function oxydases (MFO) system* represents a highly sensitive method. The MFO response to a xenobiotic is examined by evaluation of its functional components, mostly: *P450 cytochrome*, *ethoxyrheorufin-O-deethylase* (EROD), *ethoxycumarin-O-dealkylase* (ECOD), *arylhydrocarbon hydroxylase* (AHH) and *NADPH-cytochrome-c-reductase*. The activity of these enzymes is commonly established by fluorescence, luminiscence, absorption or immunoassays. It was revealed that Zn and Cu generate an increased activity of the cytochrome P4501A (CYP1A) monooxygenase in *Aporrectodea tuberculata* sp., and that the activity of this enzyme represents an efficient biomarker in evaluation of soil contamination [8]. Saint-Dennis et al. showed that the enzymes *methoxyrheorufin-O-deethylase* (MROD), NADH and NADPH cytochrome-reductase are valid biomarkers in testing soil contamination with Pb in earthworm species *E. fetida andrei* [13].

Glutathione-S-transferase (GST) represents another detoxification enzyme, well-studied in terrestrial non-vertebrates. The role of GST in phase II reactions is to detoxify electrophile intermediate compounds by conjugation with glutathione. Several xenobiotics induce enhanced activity of the GST system in exposed organisms. Lukkari observes a significant correlation between GST activity and the metal concentration in *Aporrectodea tuberculata* exposed to Zn and Cu, and concludes that GST appears to represent an effcient biomarker in monitoring soil contamination with these metals [8]. Similar effects were obtained after *E. fetida andrei* exposure to Pb [13].

Testing stress-responsive proteins is based on the induction of stress protein synthesis as a result of protein denaturation (proteotoxicity) caused by exposure to contaminants. Heat shock proteins (HSP) are proteins with variable molecular weight, identified by experiments in *Drosophila melanogaster* to be involved in the adaptative response to high temperatures. Currently, they are also termed *stress proteins* (SP). Modern methods used to investigate stress protein expression include Western blotting, the reverse-transcription polymerase chain reaction (RT-PCR), ELISA and transgenic animals studies. Of these, 1-D and 2-D Western blotting is the most frequently used method to evaluate proteotoxicity. The transfer of earthworms from a non-polluted soil to a soil contaminated with heavy metals rapidly re-sults in enhancement of HSP-60, -70 and -90 expression in the species *Lumbricus rubellus*. Exposure of the species *E. Foetida* and *L. rubellus* in soils contaminated with Zn, Cu, Pb and Cd results in active expression of stress-responsive proteins HSP -70 and HSP-72 [6]. Nadeau proposes that expression of HSP in these species represents an early biomarker of soil contamination with Pb, Cd, Cu and Hg [10]. These conclusions are confirmed by Brulle [1] who recommends the use of HSP expression in *E. foetida* sp., as a biomarker of exposure to Cd.

Metallothioneins (MT) and *metal-binding-proteins* (MBPs) have their role not fully elucidated but, generally, it is considered that these proteins are involved in the intracellular binding of essential and non-essential metals in tissues. MTs represent cysteine-rich proteins with low-molecular weight, with high affinity for heavy metals such as Hg, Ag, Zn, Ni, Co, Cu and Cd. The metals are bound by a protein through sulphur atoms found in the structure of the aminoacid cysteine. The metal is fixed in the proteic region characterized by the presence of three cysteine residues that may fix a metal atom. In the majority of species, Zn, Cd or Cu are the most potent inductors of MT synthesis. MTs and other MBPs are found in several animal species, plants and eukaryotes. The earthworms present a high bioaccumulation capacity of several metals such as Cd, Zn and Cu [1, 11, 18]. For example, the concentration of MT significantly increases in the species *Aporrectodea tuberculata* after exposure to Cu and Zn [8]. In *E. foetida*, high MTs activity was linked to exposure to Zn, Cu, Pb and Cd [6].

REFERENCES

[1] Brulle F., Mitta G., Cocquerelle C., Vieau D., Lemière S., Lepretre A., Vandenbulcke F.: Cloning and real-time PCR testing of 14 potential biomarkers in *Eisenia fetida* following cadmium exposure, Environ. Sci. Technol., 2006, 40: 2844-2850.

[2] Davey J.C., Bodwell J.E., Gosse J.A., Hamilton J.W.: Arsenic as an endocrine disruptor: Effects of Arsenic on estrogen receptor–mediated gene expression *in vivo* and in cell culture, Toxicol. Sci., 2007, 98: 75-86.

[3] Gastaldi L., Ranzato E., Capri F., Hankard P., Peres G., Canesi L., Virarengo A., Pons G.: Application of a biomarker battery for the evaluation of the sublethal effects of pollutants in the earthworm *Eisenia Andrei*, Comp. Biochem. Physiol. C Toxicol. Pharmacol., 2007: 398-405.

[4] Georgescu B., Iozon D., Helgiu A., Mierlita D.: The fertilizing value of the ecological mannure obtained as a result of the womcomposting process by using the *Eisenia foetida* species, Bull. USAMV Cluj-Napoca, seria Zootehn. Biotehnol., 2002, 57: 310-313.

[5] Homa J., Niklinska M., Plytycz B.: Effects of heavy metals on coelomocytes of the earthworm

Allolobophora chlorotica, Pedobiologia, 2003, 47: 640–645.

[6] Homa J., Olchawa E., Stürzenbaum S.R., Morgan A.J., Plytycz B.: Early-phase immunodetection of metallothionein and heat shock proteins in extruded earthworm coelomocytes after dermal exposure to metal ions, Environ. Pollut., 2005, 135: 275–280.

[7] Hopkin S.P.: Ecophysiology of metals in terrestrial invertebrates. London UK: Elsevier Applied Science, London, UK, 1989: 366 pp.

[8] Lukkari T., Taavitsainen M., Soimasuo M., Oikari A., Haimi J.: Biomarker responses of the earthworm *Aporrectodea tuberculata* to copper and zinc exposure: differences between population with and without earlier metal exposure, Environ. Pollut, 2004, 129: 377–386

[9] Malley C., Nair J., Ho G.: Impact of heavy metals on enzymatic activity of substrate and on composting worms Eisenia foetida, Bioresour Technol, 2006, 97: 1498–1502.

[10] Nadeau D., Corneau S., Plante I., Morrow G., Tanguay R.M. Evaluation for Hsp70 as a biomarker of effect of pollutants on the earthworm *Lumbricus terrestris*, Cell Stress Chaperones, 2001, 6(2): 153-163.

[11] Ndayibagira A., Sunahara G.I., Robidoux P.Y.: Rapid isocratic HPLC quantification of metallothionein-like proteins as biomarkers for cadmium exposure in the earthworm *Eisenia andrei*, Soil. Biol. Biochem., 2007, 39**:** 194–201.

[12] Ricketts A., Morgan A.J., Spurgeon D.J., Kille P.: Measurement of annetocin gene expression: a new reproductive biomarker in earthworm ecotoxicology, Ecotoxicol. Environ. Saf., 2004, 57: 4-10.

[13] Saint-Denis M, Narbonne J.F, Arnaud C, Ribera D. Biochemical responses of the earthworm Eisenia fetida andrei exposed to contaminated artificial soil: effects of lead acetate, Soil Biology & Biochemistry, 2001, 33: 395-404.

[14] Sanchez-Hernandez J.C.: Earthworm biomarkers in ecological risk assessment, Rev. Environ. Contam. Toxicol., 2006, 188: 85-126.

[15] Siekierska E., Brezozowa M. Changes in primary and secondary spermatocytes in seminal vesicles in the earthworm *Dendrobaena veneta* (Rosa) after 10 days of cadmium exposure, Acta Biologica Cracoviensia, 2008: 68-69.

[16] Spurgeon D.J., Svendsen C., Rimmer V.R., Hopkin S.P., Weeks J.M.: Relative sensitivity of life-cycle and biomarker responses in four earthworm species exposed to zinc, Environ. Toxicol. Chem., 2000, 19: 1800-1808.

[17] Strumylaite L., Bogusevicius A., Abdrachmanovas O., Baranauskiene D., Kregzdyte R., Pranys D., Poskiene L.: Cadmium concentration in biological media of breast cancer patients, Breast. Cancer Res. Treat., 2011, 125: 511-517.

[18] Stürzenbaum S.R., Winters C., Galay M., Morgan A.J., Kille P.: Metal ion trafficking in earthworms. Identification of a cadmium-specific metallothionein, J. Biol. Chem., 2001, 276: 4013–34018.

[19] Svendsen C., Spurgeon D.J., Hankard P.K.,Weeks J.M.: Lysosomal membrane stability measured by neutral red retention - is it a workable earthworm biomarker?, Ecotoxicol. Environ. Saf., 2004, 57: 20-29.

[20] Tan S., Mahaffey K.: OPPTS/OSCP/USEPA. Evidence for mercury as an endocrine disruptor: an overview of the Literature, 2003: http://www.scribd.com/doc/1790574/ Environmental-Protection-Agency-tan-s.

[21] Takiguchi M., Yoshihara S.: New aspects of cadmium as endocrine disruptor, Environ. Sci., 2006, 13: 107-116.