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Earthworm biomarkers: The new tools of environmental impact assessment

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ABSTRACT

Due to their position at different levels in food webs, invertebrates play functional key-roles in ecosystems and when affected by chemicals, they can be responsible for dramatic changes in community structure and function. Therefore, they display a great potential for evaluating the ecological impact of pollutants. Biomarkers in soil invertebrates like that of earthworms are regarded as one of the most rapidly developing fields in ecotoxicological research. They are used for both qualification and quantification of exposure and adverse effects of various environmental pollutants mainly of chemical stress. Soil pollution has enormously increased during the last decades due to the intensive use of biocides and fertilizers in agriculture, industrial activities, urban waste and atmospheric deposition, leading to degradation by which the soil losses it's agronomic and environmental qualities, as well as flora and fauna residing in it. Soil invertebrates like earthworms offer meaningful targets because they play major role in the functioning of the soil ecosystem by enhancing soil structure and the decomposition of organic materials. 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. These features, among others, have led to the popularity of earthworms as excellent bioindicators of soil pollution. In ecotoxicology of earthworm, there exist a range of biomarkers of toxic compounds. The present communication will highlight the use of earthworm biomarkers to have impact assessment of toxicological compounds in use.

KEY WORDS: HERBICIDES, INVERTEBRATES, BIOMARKERS, ECOTOXICOLOGY, TOXICO SIMULATION

INTRODUCTION

The main thrust of ecotoxicological work is to provide information to estimate ecological risk. Biomarkers are used as a sensitive, "early warning" tool for impact assessment of various contaminants on terrestrial ecosystem. The term biomarkers has been defined as any functional measure of exposure that is characterized at a suborganism level of biological organizations (Adams *et al*., 2001). The National Research Council of US (NRC, 1987, 1989) defines biomarkers as "Indicators of events in biological systems or samples" and was further described as "tools that can be used to clarify the relationship, if any, between exposure to a xenobiotic substance and disease". Also, the NRC and WHO (2001) classified biomarkers into three categories based on their relation to the exposure-disease continuum: biomarkers of exposure, effect and susceptibility. Good biomarkers are sensitive indices of both pollutant bioavailability and early biological responses.The success of biomarkers in supporting ecological risk assessment depends importantly on the identification of valid biomarkers and the establishment

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of process-level linkages between biomarkers and higher-level responses (Adams, 2003).

Soil is a complex mixture of mineral matter, organic matter and its fauna. Management of soil quality i.e. fertility and functioning of tropical ecosystem is dominantly depends upon its soil fauna which are the main consumers and decomposers of the soil ecosystem (Handrix, 2000) Recently, growing interests on soil fauna have made them attractive model for monitoring adverse ecological impact of pollutants. The plentifulness or activity of soil macrofauna has been used as an indicator for evaluating the biological health of soils (Bauble and Schmidt, 1997). Earthworms are excellent bio-indicator of relative health of ecosystem among other terrestrial invertebrates, as they possess a number of qualities that predispose them for use in environmental monitoring and remediation of soil (Reinecke and Reinecke, 2004; Ricketts *et al*., 2004). A wide range of biomarkers have been developed in earthworms like behavioral, reproductive, enzymological, lysosomal, genetic, immunological, neurological and histopathological, including the effect of both organic and inorganic substances (Ali *et al*., 2012).

Soil pollution has enormously increased during the last decades due to the intensive use of biocides and fertilizers in agriculture, industrial activities, urban waste and atmospheric deposition. Its occurrence is related to the degree of industrialization and intensity of chemical usage. Soil pollution causes decrease in soil fertility, alteration of soil structure, disturbance of the balance between flora and fauna residing in the soil, contamination of the crops, and contamination of groundwater, constituting a threat for living organisms. The most diffusive chemicals occurring in soil are heavy metals, pesticides, petroleum hydrocarbons, polychlorobiphenyl (PCBs), dibenzo-p-dioxins/diben-zofurans (PCDD/Fs).

The popularity of earthworms as excellent bioindicators make them a robust model for assessing soil pollution. In ecotoxicology of earthworm, there exist a range of biomarkers of toxic compounds, including biomarkers from the molecular to the organismal level like metallothioneins, stress proteins, cholinesterases, detoxification enzymes, parameters of oxidative stress and others. Measurement of biomarkers in invertebrates or earthworms certainly provides valuable information on both the ecological impact of long-term chemical contamination (diagnostic approach) and the conditions of biological restoration of damaged ecosystems (predictive approach). The present communication will highlight the use of earthworm biomarkers to have impact assessment of toxicological compounds in use.

TYPES OF BIOMARKERS

Many metals are essential to living organisms but some of them are highly toxic or become toxic at high concentrations, these include iron (Fe), Copper (Cu), Zinc (Zn), Cobalt (Co), Molybdenum (Mo), and Manganese (Mn). Light metals such as Sodium (Na), Potassium (K), and Calcium (Ca) play important biological roles. Metals such as Mercury (Hg), Lead (Pb), Nickel (Ni), Chromium (Cr), Cadmium (Cd), and Arsenic (As) are generally not required for metabolic activity and are toxic to living organisms at quite low concentrations (Ali, 1998; Valavanidis and Vlachogianni 2010).

When characterizing toxicological responses, it is desirable to distinguish each step in this continuum. Biomarkers signify these alterations in biological systems and may be indicators of exposure, effect or susceptibility and may overlap sometimes (Perera 1996, Perera and Weinstein 2000, Jakubowski and Trzcinka-Ochoka 2005, Nordberg 2010).

Biomarkers of exposure:

"An exogenous substance or its metabolite or the product of an interaction between a xenobiotic agent and some target molecule or cell that it is measured in a compartment within an organism". These types of biomarkers are also known as "biological dosimeters" or biomarkers of internal dose, and when they measure the product of the interaction with target molecules they are regarded as "biomarkers of biological effective dose" (Timbrell 1998).

Biomarkers of effect:

"A measurable biochemical, genetic, physiological, behavioral or other alteration within an organism that, depending on the magnitude, can be recognized as associated with an established or early health impairment or disease" (Timbrell 1998).

Biomarkers of susceptibility:

"An indicator of an inherent or acquired ability of an organism to respond to the challenge of exposure to a specific xenobiotic substance" (Pavanello and Clonfero 2000, Sakai 2000).

Earthworm: An Excellent Bioindicator of Soil Pollution

A greater proportion (>80%) of biomass of terrestrial invertebrates is represented by earthworms which play an important role in structuring, drainage, aeration and increasing the nutrient content of the soil. In addition , earthworms directly and indirectly influence soil microbial communities, mainly through the process of feeding, burrowing and fecal (cast) decomposition (Ali *et al*., 2006). These are large, relative immobile, numerous, easy to sample and easily identified. As they are in full contact with the substrate in which they live and consume large volumes of this substrate, earthworms are exposed to chemicals present in their terrestrial environment which not only kill them but their growth, fecundity and behavior is also affected.

Earthworms accumulate some chemicals in their tissues at higher level than that of the substrate in which they live (Hillel, 1971; Ali *et al*., 2002). These characteristics make earthworms attractive for monitoring the impact of contaminants in soils. Earthworms are also prey to many vertebrates and provide a route through which contaminant may be transferred to higher tropic levels (Ali *et al*., 2007). For this reasons, and because of their importance as stimulators of microbial decomposition process, they can be suitable bioindicators of chemical contamination of the soil in terrestrial ecosystems providing an early warning of deterioration in soil quality (Ali *et al*., 2009; Fischer and Koszorus, 1992; Culy and Berry, 1995; Sorour and Larink, 2001; Bustos-Obreg and Goicochea, 2002).

Biomarkers in Earthworms for Impact Assessment of Environmental Pollutants

More recently the use of biomarkers to estimate either exposure or resultant effects of chemicals have received considerable attention. Biomarkers in theory offer two outstanding advantages in ecotoxicological investigations. First, by their very nature they are responsive only to the bio or toxicoactive fraction of the accumulated body burden of one or more potential toxicants; to this extent they reflect, albeit indirectly, the analytically elusive bioavailability fraction of environmental chemicals within chosen test organism. Secondly they integrate the interactive effects of several chemicals in complex mixture (Weeks, 1995).

Biomarkers used as measures of toxic effects in organisms at the level below individual (molecular or cellular) level (Van Gestel and Van Brummelen, 1996), they represent early or initial responses to environmental perturbation and contamination. In ecotoxicology of earthworm, there exist a range of biomarkers of toxic compounds, including biomarkers from the molecular to the organismal level like metallothioneins, stress proteins, cholinesterases, detoxification enzymes, parameters of oxidative stress and others (Novais *et al*., 2011). Besides, the investigation of new, potent biomarkers in earthworms is in accelerating phase for the impact assessment of soil pollutants.

Biomarkers of Toxic metal exposure

Potential metal contaminants are produced from various industries such as mining, metal, pigment and chemical manufacturing as well as in combustion of fossil fuels, including the exhaust emission of motor vehicles. In the field of toxicology, it is essential to be able to measure the exposure to a toxic agent, the extent of any toxic response and also to predict the likely effects. Hence, integrating measures of different types of responses to toxic stress of exposed individuals and populations, offers a powerful tool for documenting the extent of exposure and the effects of environmental metal contamination. Therefore, use of biomarkers for environmental monitoring of individuals and populations exposed to chemical pollution has gained much attention in the last decades, because it offers great opportunities for a fast and sensitive detection of chemical stresses within organisms (Peakall and Shugart, 1992; Ali, 1997; Ali, 1998; Handy *et al*., 2003).

Metallothioneins

Metallothioneins (MTs) are low-molecular-weight cysteinrich metal-binding proteins that are involved in homeostasis of essential metals like Cu and Zn and detoxification of non essential metals such as Ag, Cd and Hg (Costello *et al*., 2004; Amiard *et al*., 2006). Toxicological effects of heavy metals can be countered by detoxification mechanisms. The ability of relatively low exposure to non-essential trace metal contaminants to induce metallothionein has generated interest in the use of metallothionein as a biomarker for metal pollution. Induction of metallothionins due to metal exposure has been observed in various organism including earthworms (Strzenbaum *et al*., 2001). Significant induction of MTs has been reported by researchers on different species of earthworms for example *Lumbricus rubellus, Eisenia fetida, Eisenia andrei* exposed to cadmium (Calisi *et al*., 2009; Demuynck *et al*., 2006; Ndayibagira *et al*., 2007; Brulle *et al*., 2007), or in *Lumbriucus terrestris* exposed to cadmium, copper and mercury (Khan *et al*., 2007; Calisi *et al*., 2011) and in *Lumbricus mauritii* exposed to Pb and Zn contaminated soil (Maity *et al*., 2011). It is well known fact that earthworms have high degree of tolerance to heavy metal exposure. Metallothenin is the most videly used biomarkers in earthworms for the early detection of exposure to heavy metals in soil monitoring.

Hemoglobin oxidation

Changes in haematology are reported to be early warning signals of the toxic effects of pollutants in vertebrates (Dauwe *et al*., 2006), but they are poorly explored in invertebrates and for comparison in earthworms. Earthworms have a closed circulatory system. Blood contains haemoglobin which is a large extracellular hemoprotein flowing in a closed circulatory system. In spite of the fundamental role of this respiratory pigment in earthworm physiology, little is known about its sensitivity to environmental pollutants.

Recently Calisi *et al*., (2011) demonstrated heavy metal (cadmium, copper, mercury) exposure to significantly induce changes in either Hb concentration or its oxidation state in the earthworm *Lumbricus terrestris*. Exposure to heavy metals (Cd, Hg, and Cu) was found to increase blood Hb concentration. In addition to changes in the Hb concentration, heavy metals showed a dramatic effect on the oxidation state of the respiratory pigment. Future studies will be addressed to evaluate if the observed response is specific for heavy metal exposure or represents a biomarker of general health of earthworms in polluted sites. In any case it demonstrated to be a suitable biomarker of exposure/effect to be included in a multibiomarker strategy in earthworm in soil monitoring assessment.

Biomarkers of Pesticides Exposure

Nowadays it is widely accepted that current agricultural practices cause a loss of biodiversity. Moreover, the introduction of vast areas of monocultives (e.g., biofuel crops) contributes to increase the risk for crop loss by pest infestation (Ali *et al*., 2006). Plant protection products (PPPs) are still necessary for combat pests. The massive use of pesticides leads to a set of environmental hazards on non-target organisms of ecological and agronomic concerns such as earthworms, pollinators or natural enemies of pests. Moreover, the occurrence of pesticide residues in soil can change microbial communities and soil enzyme activities involved in nutrient cycles. These effects can lead, in turn, to a loss of soil quality. Biomarkers are often used to provide mechanistic understandings on the toxic effects observed at the whole-organism level. Classical biochemical biomarkers have been used in terrestrial invertebrates, mainly earthworms, to assess exposure to organophosphate (OP) and carbamate (CM) pesticides.

Acetylcholinesterase

Acetylcholinesterase (AChE) is a key enzyme in the nervous system, terminating nerve impulses by catalyzing the hydrolysis of neurotransmitter acetylcholine (Thompson and Richardson, 2004). AChE is the target site of inhibition by organo-phosphorus and carbamate pesticides. In particular, organophosphorus pesticides inhibit the enzyme activity by covalently phosphorylating the serine residue within the active site group. They irreversibly inhibit AChE, resulting in excessive accumulation of acetylcholine, leading to hyperactivities and consequently impairment of neural and muscle system. The monitoring of acetylcholinesterase activity in the brains of fish and birds in the field has become a technique commonly used for diagnosing the exposure to cholinergic poisons (Greig-Smith, 1991; Zinkl *et al*., 1991). Acetylcholinesterase represents the main cholinesterase in earthworms (Rault *et al*., 2007).

AChE inhibition in earthworms is presently regarded as giving early warning of adverse effects of pesticides (Booth and O'Halloran, 2001), and consistently included among the batteries of biomarkers employed for early assessments of pollutant impact on wildlife in terrestrial ecosystems. However, concerning AChE in earthworms only a few pesticides in use have been tested against relatively few earthworm species both in laboratory tests and under field conditions (Rao *et al*., 2003; Calisi *et al*., 2009; Scott-Fordsmand and Weeks, 2000; Rao and Kavitha, 2004; Gambi *et al*., 2007). As pointed out by Scott-Fordsmand and Weeks (2000) the potential use of AChE in earthworms as biomarker of pesticide exposure has not been sufficiently explored.

Glutathione S-transferase (GST) and Antioxidant enzymes.

Many agrochemicals such as OP insecticides are able to induce oxidative stress (Lukaszewicz-Hussain, 2010), a situation in which the production of reactive oxygen species (ROS) overcomes the cellular antioxidant mechanisms (molecular and enzymatic), leading to the oxidative damage of biomolecules (e.g., lipids, proteins or DNA). Glutathione level is one of the most used biomarker of pro-oxidant exposure in fish (van der Oost *et al*., 2003) and birds (Koivula and Eeva, 2010). Glutathione transferases (GSTs) form a ubiquitous superfamily of multi-functional dimeric enzymes (w50 kDa) with roles in detoxification. Several studies have been concerned with changes in glutathione concentration and glutathione-dependent enzymes in terrestrial invertebrates i.e. earthworm on metals and pesticide exposure (Aly and Schröder, 2008; Maity *et al*., 2008; Lukkari *et al*., 2004; Saint-Denis *et al*., 2001; Booth *et al*., 2000). Biomarkers of oxidative stress have been mainly explored in earthworms exposed to, or inhabiting in, metal-polluted environments. For example, earthworm GST activity is a noteworthy detoxication system (Stenersen, 1984), which is induced in earthworms exposed to organochlorine pesticides (Hans *et al*., 1993). However, no effects on this enzyme activity were observed in earthworms exposed to the OP fenitrothion (Booth and O'Halloran, 2001) or the CM carbaryl (Ribera *et al*., 2001). Herbicides also induce the GST activity of earthworms.

Cellular Biomarkers

Coelomic fluid of earthworm shows very interesting features from ecotoxicological point of view for the development of novel cellular biomarkers. It helps in transport of pollutant in the exposed organism (Engelmann *et al*., 2004). Five cell types were observed by Calisi *et al*., (2009) in *Eisenia fetida* celomic fluid, corresponding to the previously described coelomocyte cell types (Valembois *et al*., 1985): leukocytes type I (basophilic) and II (acidophilic), granulocytes,-neutrophils, and eleocytes. Immunoactive coelomo-cytes and their viability is one of the most promising surrogate assays to assess immunotoxic risks (Bunn *et al*., 1996; Burch *et al*., 1999).

Several studies concerned with the effects of environmental pollution including heavy metals on earthworm immune functions mediated by coelomocytes (Scott-Fordsmand and Weeks, 2000).

Fungere *et al*., (1996) recorded the inhibition of phagocytic activity of coelomocytes exposed in vitro on heavy metal (Cd, Zn) solutions. Homa *et al*., (2003) studied the effects of heavy metal toxicity on the coelomocytes of the earthworm *A. chlorotica* and reported that the coelomocytes viability and activity was significantly reduced. This was explained as a result of accumulation of heavy metals in the body tissues of the earthworms including coelomocytes which in turn affects their and viability.

In earthworms, lysosomal fragility as a result of heavy metal toxicity has been developed as a promising biomarker by Weeks and Svendsen (1996). The method used is the neutral red retention assay (NRR-assay), a histochemical technique based on the principle that only healthy lysosomes can retain the acidotrophic, red dye after an initial uptake. The time lysosomes are able to retain the dye is called neutral red retention time (NRR-time) and the NRR-time decreases when lysosomes are exposed to membrane disrupters such as heavy metals. The NRR-assay has earlier been used to assess the effects of copper, cadmium, nickel and zinc on earthworm coelomocytes (Weeks and Svendsen, 1996; Svendsen and Weeks, 1997a,b; Harreus *et al*., 1997; Scott-Fordsmand *et al*., 1998; Reinecke and Reinecke, 1999; Scott-Fordsmand *et al*., 2000; Spurgeon *et al*., 2000; Gupta, 2000).

Biomarkers of Genotoxicity

Coelomocytes of earthwaorms coelomic fluid is also taken for monitoring the genotoxic effect of soil pollutants in earthworms. Many pollutants in soil like metal etc. may cause its deleterious effect on the structure of DNA, thus it is an important indicator for evaluation of dreadfull effects of pollutants on earthworm health (Frenzilli *et al*., 2001; Reinecke and Reinecke 2004). Hence, the evaluation of metal sublethal toxicity should always include biomarkers of DNA damage because such damage may result in inappropriate gene expression and, subsequently, in more concerning genotoxic and mutagenic effects (Hartsock *et al*., 2007). The single cell gel electrophoresis (or comet assay) and micronucleus test are two most extensively used methods in the detection of genotoxicity of chemicals in the environment. The single cell gel electrophoresis assay (also known as the Comet assay) which is a simple, rapid and sensitive technique for analysing and quantifying DNA damage in individual cells, as single- and double-strand breaks, alkali-labile sites, oxidative DNA base damage (Cotelle and Ferard, 1999). The Comet assay is an effective method for determining DNA damage levels in the coelomocytes of earthworms exposed to genotoxic compounds, both *in vivo* and *in vitro*, in several studies (Reinecke and Reinecke, 2004).

Instead of comet assay micronucleus assays have emerged as one of the preferred methods for assessing chromosome damage because they enable both chromosome loss and chromosome breakage to be measured reliably accumulated during lifespan of the cell in vertebrates and invertebrates. Sforzini *et al*., (2010) provided the first step of validation of this test on earthworm (*Eisenia andrei*) cells.

CONCLUDING REMARKS

Most biomarkers provide an indication of pollutant exposure. Earthworm biomarkers have become increasingly relevant for the evaluation of contaminants effects on soil organisms. These organisms are considered suitable indicators of environmental change in agricultural environments. Biomarkers related to metal exposure (MT induction) have been extensively investigated. Other biomarkers (e.g., ChE) commonly used in biomonitoring programs with vertebrates have received little attention in earthworm studies. Additional studies with different species of earthworm, including different endpoints, temperature regimes and soil types, are required. Research should be extended to ecologically relevant species of earthworms, as stated earlier and also to other soil fauna to get a comprehensive knowledge on the malfunction in the soil biological processes due to soil pollutants. So, there is a need to acquire more knowledge on the chemical nature, mode of action, and means of degradation of pollutants in soil, so that harm caused to soil fauna as well as to organisms higher up in the food chain can be minimized.

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