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Bioindicators and Biomarkers in the Assessment of Soil Toxicity

Carmem Silvia Fontanetti, Larissa Rosa Nogarol, Raphael Bastão de Souza, Danielli Giuliano Perez and Guilherme Thiago Maziviero
*Department of Biology, Institute of Biosciences, São Paulo State University (UNESP)
Rio Claro, SP,
Brazil*

1. Introduction

Several potentially harmful chemical compounds, derived from activities of urban centres, rural properties and industries are constantly released into the terrestrial environment. In this context, the scientific community has shown increasing interest in the detection, knowledge and control of environmental agents responsible for damages to human health and sustainability of ecosystems (Magalhães & Ferrão-Filho, 2008).

Monitoring the types and quantities of toxic substances that are entering into the terrestrial environment is an exhaustive and problematic task due, mainly, to the complexity and cost resulting from the identification of the chemical substances involved. Despite the numerous analytical methods available, collecting sufficient samples in a timely fashion continues to be a great obstacle in the evaluation of environmental damages (Silva et al., 2003).

Furthermore, the determination of isolated substances by traditional chemical analyses has a limited environmental application, since it does not detect the effects on the organisms neither inform about the possible interactions between the substances (additive, antagonistic or synergistic), as well as their bioavailability (Magalhães & Ferrão-Filho, 2008). In this sense, researchers have pointed the necessity to apply biological methodologies in order to obtain an ecosystemic approach.

Biological factors may indicate better the environmental balance through the biotic indexes, derived from the observation of bioindicator species. According to Hodkinson and Jackson (2005), it is called bioindicator a species or group of species that reflects biotic and abiotic levels of contamination of an environment, presenting alterations that enables the generation of information about the quality of the environment, for example, accumulating substances in concentrations higher than those considered normal or essential for its body metabolism or presenting alterations in the number of organisms. Such organisms, due to their characteristics of little ecological tolerance to some chemical substances, can present some alteration, whether it is physiological, morphological of behavioural, when exposed to certain pollutants (Magalhães & Ferrão-Filho, 2008).

Due to their close contact with soil, some taxonomic groups of invertebrates belonging to the meso- and macro-fauna such as, Isopoda, Collembola, Oligochaeta and Diplopoda, have been proposed as bioindicator organisms (Hopkin, 2002). In addition, higher plants such as *Allium cepa* (onion), *Arabidopsis thaliana* (mustard), *Hordeum vulgare* (barley), *Tradescantia sp.*, *Vicia faba* (broad bean) and *Zea mays* (corn) are also commonly used in the assessment of soil toxicity, particularly for cytotoxicity, genotoxicity and mutagenicity assays (White & Claxton, 2004).

In a biological system, the sequential order of alterations promoted by the presence of pollutants occurs in crescent levels of biological organization, extending from the molecular or biochemical level to the physiological or individual level, until the population and ecosystem level (Stegeman et al., 1992). When a significative alteration is evident, the ecosystem must be already severely damaged. Therefore, techniques that show responses at lower levels of biological organization are considered more preventive (Nascimento et al., 2008).

Morphological alterations can be used as biomarkers in toxicity investigations of specific chemical compounds and in the monitoring of the acute and chronic effects of organisms exposed to impacted environments. In this context, the morphological analysis of target organs, carried out by ultra-morphology, histology and ultrastructure, has become widely used in studies with invertebrates, aiming to identify different damages caused by harmful substances to the organisms (Fontanetti et al., 2010).

Another tool that has shown to be increasingly efficient in the assessment of soil toxicants on the organisms is the use of molecular biomarkers. Recent studies show great interest in the use of enzymatic biomarkers as a form to monitor the environment, since the increase or inhibition of the activity of certain enzymes can explain a possible response to the environment stress.

Due to the importance to ensure the genetic integrity of the organisms, biomarkers of genotoxicity are gaining attention in the evaluation of the toxic potential of soil samples (Misik et al., 2011). The tests used in the genotoxic assessment of an agent (genotoxicity and mutagenicity tests), include the Ames test, chromosome aberrations test, micronucleus test, comet assay, SMART test (Somatic Mutation and Recombination Test), microarray and microscreen, using techniques of cellular, molecular and genetic biology both *in vitro* and *in vivo*, *in situ* and *ex situ*.

Given the above, the aim of the present chapter was to compile and discuss information present in the literature about the use of animal and plant bioindicators in the analysis of soil toxicity, as well as characterize the different biomarkers used in these organisms that enable the assessment of the soil toxicant effects in different levels of the biological scale, i.e., morphological, biochemical and genotoxic.

2. Complex substances, organic compounds and metals: potential soil contaminants

Population growth combined with the increasing industrialization is responsible for generating tons of waste per day, which, many times, are accumulated in the environment without any previous treatment. Soil becomes a cheaper and practice alternative for the final disposal of these residues, but not without consequences. Soil contamination is a broad problem, since the contaminants can be leached into groundwater, rivers and lakes. The major xenobiotics responsible for the contamination of this compartment as well as their implications for invertebrates and plants will be discussed.

2.1 Vinasse

Among the substances released into the soil with toxic potential it can be cited the vinasse, a product of the alcohol production, composed by water (97%) and a solid fraction (organic matter and mineral elements). According to Sahai et al. (1985), due to the fast growth of the distilleries, there was, consequently, an increase in the amount of this residue, which was traditionally discharged in open areas or next to water courses, causing air, water and soil pollution. For Junior et al. (2008), the reuse of previously treated vinasse as fertilizer or soil conditioner becomes an alternative of great interest. However, Lyra et al. (2003) affirm that there are only few studies that evaluate its pollutant potential on soil and groundwater.

Thus, studies about the effects of vinasse application in the soil have been developed by researchers of different countries. Recently, Brazilian researchers have studied the genotoxicity and mutagenicity of vinasse applied *in natura* in the soil or associated with other compounds. Souza et al. (2009) used *A. cepa* to evaluate the mitotic and chromosome abnormalities resulted from exposure to landfarming soil treated with vinasse, used as a possible bioremediator. According to the authors, the vinasse was responsible for potentiating the clastogenicity of the landfarming by decreasing the pH and, thus, making available the metals that were strongly adsorbed in the organic matter of the soil.

Other studies on the vinasse toxicity were conducted by Christofoletti and Fontanetti (2010) and Pedro-Escher and Fontanetti (2010) also using *A. cepa* as test organism. In the first study, the preliminary results show that the vinasse did not present cytotoxicity nor mutagenicity, but it presented genotoxic potential when applied *in natura* or associated with sewage sludge samples; the second study showed that different concentrations of vinasse diluted in water (12.5%, 25% and 50%) presented genotoxic potential and only the raw vinasse presented mutagenic potential, thus suggesting that the vinasse can cause damages in the genetic material of certain organisms.

2.2 Sewage sludge

Another residue with pollutant potencial and problems in final disposal is the sewage sludge generated in the STSs (Sewage Treatment Stations). Its great production, mainly in the large urban centres, has led researchers to intensify the studies about the use of these waste with agricultural purposes. Therefore, recycling, via agriculture use, presents itself as a global trend (Lopes et al., 2005).

Nevertheless, sewage sludge can present, in its composition, undesirable chemicals (metals and organic chemical compounds) and biological elements (pathogens) that, in contact with man and/or fauna and flora, may cause contamination and diseases. Thus, any decision on the most appropriate final destination depends on the evaluation and minimization of the contamination risks of the environment and man (Rocha & Shirota, 1999).

Studies involving millipeds exposed to sewage sludge have shown that its components can affect the integrity of organs such as the midgut of these animals (Godoy & Fontanetti, 2010; Nogarol & Fontanetti, 2010, 2011; Perez & Fontanetti, 2011a). Mazzeo et al. (2010), using *A. cepa,* investigated the genotoxic and mutagenic potential of domestic sewage sludge at different concentrations.

2.3 Polycyclic aromatic hydrocarbons (PHAs)

Some of the main pollutants that cause concern in relation to soil contamination are the PAHs (Bispo et al., 1999), which are compounds formed by two or more benzene rings, exclusively constituted by atoms of carbon and hydrogen (Netto et al., 2000). There are many origin sources but it can be highlighted industrial processes, such as petroleum refining, combustion of organic matter and burning of coal (Page et al., 1999). According to the IUPAC (International Union of Pure and Applied Chemistry) there are, currently, over

100 known PAHs, however only 16 have environmental and toxicological importance (Environment Protection Agency [EPA], 1986). These compounds are able to react, directly or after undergoing metabolic transformations, with the DNA, becoming potential carcinogens and efficient mutagens.

Soil receives considerable amounts of PAHs that, because of the complexity of their chemical structure, low solubility in water and strong sorption tendency into the soil, become recalcitrant and remain for long periods in the environment, enhancing the probability of exposure of humans and animals to these compounds (Jacques et al., 2007). When present in the environment they can be transferred to invertebrates by ingestion of soil and plant material contaminated or by cuticle (Achazi & Van Gestel, 2003).

In order to reduce the negative impact of these compounds in the soil, petroleum refineries use a bioremediation system called landfarming. The technique has been used for the treatment of soils contaminated with hydrocarbons for 100 years and, the petroleum industry for at least 25 years (Riser-Roberts, 1998). Currently, other types of industry started to employ this technique, such as textile and food industries and treatment of effluents.

Souza et al. (2009) carried out bioassays with *A. cepa* in order to assess landfarming soil samples before and after biodegradation of hydrocarbons. Before biodegradation, the landfarming had 13.5 g/Kg of Total Petroleum Hydrocarbons (TPH) and caused strong clastogenic and mutagenic effects. After 108 days of biodegradation, the concentration of TPH decreased 27% with significant reduce of mitotic and chromosome abnormalities, micronuclei and nuclear buds.

Using the diplopod *R. padbergi* exposed to different concentrations of industrial soil contaminated with PAHs, Souza et al. (2011) and Souza and Fontanetti (2011) analyzed the perivisceral fat body and midtgut of the animals and verified that there were several alterations in these two tissues.

2.4 Dioxins

Seven dibenzo-p-dioxins (PCDDs), 10 polychlorinated dibenzofurans (PCDFs) and 12 polychlorinated biphenyls (PCBs) are called dioxins (Word Health Organizatrion [WHO], 2010; United States Environmental Protection Agency [USEPA], 2000, 2003), being released into the environment as a byproduct of chemical processes and through the combustion of industrial and municipal wastes (Stephens et al., 1995). According to Schlatter (1994), since the accident in Seveso, Italy, they became the symbol of threat caused by toxic chemicals. As a result of widespread fear, dioxins are a matter of real concern in relation to environmental contamination.

Among the isomers of PCDDs, the most toxic is 2,3,7,8 - tetrachloro-dibenzo-para-dioxin (2,3,7,8-TCDD) (Eisler, 1986). There is little information in literature on the effects of PCDDs on terrestrial invertebrates. Reinecke and Nash (1984) reported that two species of earthworms (*Allolobophophora caliginosa* and *Lumbricus rubellus*) showed no adverse effects when exposed for 85 days in soil with 5 ppm of 2,3,7,8-TCDD, but both species died at 10 ppm. Studies involving plants and PCBs have been made, since these organisms are less sensitive to PCBs and thus may be a possible route of biomagnification in various food chains (Sinkkonen et al., 1995).

2.5 Agrochemicals

The use of fertilizers and pesticides has become a common practice due to population growth, food crisis and consequent need for the increase in the agriculture production.

Within the existing agriculture model, agrochemicals are classified as one of the main chemical pollutants that are disseminated throughout the planet (Grisolia, 2005). However, there is still little information about the effects of these chemical compounds on invertebrates that occupy levels of high sensitivity in trophic chain (Mantecca et al., 2006).
Among the studies carried out with terrestrial invertebrates it was analyzed the possible alterations in the biomass (Niemeyer et al., 2006a), reproduction (Helling et al., 2000), behaviour (Niemeyer et al., 2006b), survival (Diao et al., 2007) and tissular and cellular lesions (Nasiruddin & Mordue, 1993) resulted from exposure to certain agrochemicals. Associated with the use of bioindicators and biomarkers it is also used the direct analysis of the presence of residues in soil samples by specific equipments such as the spectrophotometer or chromatograph.
Pesticides have been widely tested by bioassays with plants and positive results are usually obtained (Leme & Marin-Morales, 2009). A clear example is the *A. cepa* test successfully used in the evaluation of the mutagenic and genotoxic potential of herbicides such as trifluralin (Fernandes et al., 2007; 2009).

2.6 Metals

Heavy metals or trace metals are terms applied for a great amount of trace elements that are industrially and biologically important. From the point of view of human health, agriculture and ecotoxicology, the most worrying heavy metals are As, Cd, Hg, Pb, Ti and U. Studies involving heavy metals in ecosystems have shown that many areas near urban centres, mines and road systems have high concentrations of these elements (Alloway, 1994). Metals are highly persistent in the soil with persistence of up to thousands of years (McGrath, 1987) and can express their pollutant potential directly on the soil organisms by availability to plants and transference to the food chain, both by plants and by the contamination of superficial waters or groundwater (Chang et al., 1987).
The main anthropogenic sources of metals are fertilizers, pesticides, contaminated irrigation water, combustion of coal and oil, vehicular emissions, incineration of urban and industrial wastes and, mainly, mining and smelting (Tavares & Carvalho, 1992).
Due to their habits in the superficial layers of the soil, invertebrates of the saprophagous fauna, such as isopods, diplopods and springtails are regularly exposed to metals (Hopkin, 2002). Heikens et al. (2001) carried out a literature review to clarify the concentration of metals in terrestrial invertebrates and they concluded that the concentration in most of the groups happened in the order Pb>Cd>Cu. Afterwards, Köhler (2002) conducted a study to determine the location of these metals in the bodies of soil arthropods. The genotoxic potential of metals has also been studied by several authors using plants as test systems (Knasmüller et al., 1998; Rank & Nielsen, 1998).

3. Invertebrates of the edaphic fauna and higher plants as soil bioindicators

One important question in ecotoxicological studies refers to the choice of the bioindicator species. It will depend on its ecological and toxicological importance, facility to be maintained in laboratory, reproductive rate and sensitivity (it must be affected by several chemical agents but less affected by abiotic factors) (Römbke & Garcia, 2000). Many authors agree that the main features needed to be a good bioindicator are sensitivity, good representativeness and functional importance in the ecosystem, as well as easy collection, identification and analysis (Greensdale, 2007). In this context, some taxonomic groups of soil invertebrates and higher plants have been proposed as bioindicator organisms.

3.1 Terrestrial invertebrates

Terrestrial arthropods of the saprophagous fauna such as, Isopoda, Collembola and Diplopoda are among the most appropriate organisms to evaluate the effects of the accumulation of toxic substances present in the soil, due to their direct contact with contaminants present in it (Gräff et al., 1997; Hopkin et al., 1989). Annelids, in special the Oligochaeta, are also frequently used in toxicity tests. These invertebrates get in contact with a great variety of pollutants present in this compartment by their movement and ingestion of contaminated soil or leaf litter (Spadotto et al. 2004).

Oligochaeta are considered one of most important representatives of the edaphic macro-fauna (Kale, 1988). Several factors make earthworms excellent bioindicators of the toxicity of chemical substances in the soil, such as the knowledge already accumulated on their habitats and important trophic position of these invertebrates, which are situated in the lowest levels of the terrestrial food webs, serving as food for several animals and route of transference and biomagnification of contaminants along these webs (Andréa, 2010).

Due to their great importance in the soil, their wide distribution and all the reasons previously cited, earthworms, mainly the species *Eisenia fetida* (figure 1) and *E. andrei* were chosen for several toxicity tests for registration of agrochemicals in the regulatory agencies of several countries, including Brazil (Andréa, 2010). Other species such as *Lumbricus terrestris* and *L. rubellus* have been widely used in studies of bioaccumulation of metals (Amaral & Rodrigues, 2005; Veltman et al., 2007).

Fig. 1. Earthworm *Eisenia fetida.* (Photo: Raphael Bastão de Souza and Larissa Rosa Nogarol)

Collembola are among the most important members of the soil meso-fauna involved in the decomposition process and are vulnerable to the effects of its contamination (Bengtsson & Rundgren, 1984). Greensdale (2007) lists some favourable points in choosing Collembola as bioindicators, such as presence in all ecosystems, abundance and ease of collection in sufficient number to allow statistical analyses. Moreover, they have short life cycle, making that they respond quickly to environmental changes and, as they are in direct contact with the soil, they are more sensitive to some type of stress applied in the ecosystem.

Several studies point out this organism as bioindicator, applying different methodologies and evaluation parameter. Tests of reproduction associated to survival rates (Pedersen et al., 2009; Sverdrup et al., 2010;) and evaluation of abundance and/or diversity of species in areas that suffer some type of degradation (Sousa et al., 2004) are the most used methodologies.

Another taxonomic group used in toxicological analyses is Isopoda, one of the largest orders of crustaceans with approximately 10,000 thousand described species, mostly marine

(Schultz, 1982). Terrestrial isopods have already been used in toxicity tests of soil and the main parameters of evaluation were abundance of individuals (Faulkner & Lochmiller, 2000), reproduction rates (Niemeyer et al., 2009) and survival (Stanek et al., 2006).

Metals are the main toxic agents evaluated using Isopoda, since these invertebrates bioaccumulate these elements. In this sense, researchers have carried out studies on bioaccumulation (Blanusa et al., 2002; Hopkin et al., 1993), the cytotoxic effect of metals (Köhler et al., 1996a; Odendaal & Reinecke, 2003) and the detoxification mechanisms (Hopkin, 1990; Köhler & Triebskorn, 1998) and the terrestrial isopod *Porcellio scaber* is the most studied.

The importance of diplopods in the recycling of nutrients, aeration and fertilization of soil is frequently mentioned in the literature (Dangerfield & Telford, 1989). Due to the habits of the diplopods, colonizers of various soil layers, these animals can be greatly influenced by the deposition of metals, organic compounds and complex substances in the soil.

Most studies in the literature using diplopods as bioindicators of the soil are related to metals. However, the effect of organic pollutants and complex mixtures on these invertebrates is relatively little known (Souza & Fontanetti, 2011). In this context, the first study carried out with diplopods, as possible bioindicators, was conducted by Hopkin et al. (1985), involving the assimilation of metals by the species *Glomeris marginata*. In this study, it was verified a higher uptake of copper, zinc and cadmium by the animals collected in soils contaminated when compared to those animals collected in non-contaminated environments. The authors comment that ultrastructural studies of different organs would be necessary to understand the "metals path", particularly in the gut of these invertebrates.

Later, Triebskorn et al. (1991) exposed several invertebrates such as mites, insects and diplopods to different toxic substances and used the ultrastructural analysis in order to demonstrate the applicability of using such animals in biomonitoring. In the study carried out by Köhler et al. (1992), it was analyzed the impact of lead on the efficiency of assimilation in diplpods, submitted to different environmental conditions. The researchers used different species of diplopods and found that only *Glomeris conspersa* increased the ingestion of food containing lead when compared to a non-contaminated diet.

Recently, the toxicity assessment of complex substances was performed with the Brazilian species *Rhinocricus padbergi* (figure 2) exposed to different concentrations of sewage sludge (Godoy & Fontanetti, 2010; Nogarol & Fontanetti, 2010, 2011; Perez & Fontanetti, 2011a) and landfarming (Souza & Fontanetti, 2011; Souza et al., 2011). The histological and histochemical analysis, as well as the ultrastructural analysis, showed that such substances are toxic to the diplopod studied, since different tissular and cellular alterations were observed in the midgut and perivisceral fat body of these invertebrates.

Fig. 2. Diplopod *Rhinocricus padbergi*. (Photo: Larissa Rosa Nogarol and Raphael Bastão de Souza)

3.2 Higher plants

Plants, despite their structure and metabolic differences, can offer important information about the cytotoxic, genotoxic and mutagenic potential of substances, even when exposed in short term and offer some advantages such as low cost cultivation and easy maintenance, comparatively, to mammals (Rodrigues et al., 1997). In studies with complex mixtures, plants have also shown satisfactory results, indicating that plants are sensitive enough to detect the adverse effects of environmental samples (Majer et al., 2005).

Plants can be directly exposed to the contaminant, without any dilution or filtration of the sample (Steinkellner et al., 1999). Moreover, Grant (1994) cites other advantages of employing higher plants: (1) higher plants are eukaryotes, thus, their structure and cellular organization are similar to that of humans and it is possible to establish comparisons with animals; (2) the techniques employed for the study are relatively simple and can be performed with agility; (3) cultivation of the organisms has low cost and easy maintenance; (4) the assays can be carried out under a wide range of environmental conditions, pH and temperature; (5) higher plants can regenerate easily; (6) assays with higher plants can be used to assess the genotoxic potential of simple substances or even complex mixtures; (7) it can be used for *in situ* monitoring; (8) can be used for monitoring for several years and are highly reliable; (9) studies have shown correlations with cytogenetic assays in mammals; (10) can be used together with microbial assays to detect mutagenic metabolites (pro-mutagens); (11) genotoxicity studies with plants are presenting high sensitivity in tests with carcinogenic agents.

On the other hand, according to Majer et al. (2005), one of the limitations of using plants as bioindicators is the lack of sensitivity for certain classes of pro-mutagens such as the nitrosamines, heterocyclic amines and some classes of PAHs. In contrast, Ventura (2009) showed that the *A. cepa* system is susceptible to nitro aminobenzene, while Mazzeo (2009) observed the same effect for benzene, toluene, ethylbenzene and xilene (BTEX).

Among the higher plants, onion (*A. cepa*) is the most used plant to determine the cytotoxic, genotoxic and mutagenic effects of many substances present in the soil. Its cellular kinetics characteristic favours a rapid growth of the roots, due to the great number of cells in division. Therefore, the record of the mitotic activity and abnormalities in the cell cycle of the meristematic cells of its roots can be easily visualized (Grant, 1994). Leme and Marin-Morales (2009) affirm that the *A. cepa* test is a fast and sensitive technique to detect genoxotic and mutagenic substances dispersed in the environment.

The evaluation of genetic alteration can be also performed using different species of the genus *Tradescantia* (figure 3) through the detection of mutations induced by agents present in the air, soil and water by the analysis of micronuclei in the mother cell of the pollen grain (Trad-MCN). The species *Tradescantia* are specially indicated for direct application in regions and countries in development due to the advantages such as easy handling and relatively low maintenance cost (Shima et al., 1997).

Vicia faba is a popular material that has been widely used not only in cytological studies, but also in physiological experiments (Kanaya et al., 1994). This organism was initially used in radiobiological tests in investigation of mechanisms of formation of chromosomal aberrations by ionizing radiation (Read, 1959). Later, Kihlman (1975) developed and standardized the *V. faba* meristematic cell bioassay for analysis of chromosomal aberrations, and since then has been widely used for genotoxicity studies for evaluation of sister chromatid exchange (Kihlman & Kronborg, 1976; Kihlman & Andersson, 1984). This technique is very similar to *A. cepa* test; the method does not require sterile conditions or any material or equipment of high cost. Further details of this test are described by Kihlman (1975).

Fig. 3. *Tradescantia pallida*. (Photo: Guilherme Thiago Maziviero)

4. Biomarkers

Molecular, biochemical and physiological compensatory mechanisms can become operative in organisms after exposure to environmental contaminants. This may result in the inhibition or facilitation of one or more physiological mechanisms or functional and structural changes. In this sense, the use of biomarkers allows obtaining information about the biological effects of pollutants and mechanisms of action of xenobiotics on the fauna.

Several authors have proposed different definition for the term biomarkers. According to Lam and Gray (2003), biomarkers can be defined as biochemical, cellular or molecular alterations or physiological changes in the cells, body fluids, tissues or organs of an organism that are indicative of exposure or effect of a xenobiotic. Despite being older, the definition proposed by Depledge (1993) and Depledge et al. (1993) has a more comprehensive character and it is considered the most widely used nowadays: biomarkers are defined as adaptive biological responses to stressors, evidenced as biochemical, cellular, histological, physiological or behavioural alterations.

In the scope of measuring the toxic effects in the organisms at a cellular or molecular level, biomarkers represent an initial response to environmental disturbances and contamination. Therefore, they are generally considered more sensitive than the tests that measure these effects at higher levels of biological hierarchy, such as individual or population (McCarthy & Shugart, 1990).

Thus, during the last decades, several biomarkers have been used effectively, especially as tests for specific toxicants, since biomarkers when combined with biomonitors can create a sophisticated multiple target system to detect a variety of environmental hazards in a fast and economically feasible way, in a single test organism, helping in the establishment of priorities for action in the control of environmental pollution.

4.1 Morphological biomarkers

The detection of many classes of damage in several tissues and cellular types becomes possible by using morphological biomarkers. Such morphological alterations may provide qualitative evidences of a functional adaptation to the external environment (Meyers & Hendricks, 1985). Moreover, the qualitative assessment of such changes before the death of the organism may provide early indications of toxicity (Nogarol & Fontanetti, 2010; Triebskorn et al., 1999).

For such analysis, histology and ultrastructure are used. By these techniques it is possible to diagnose cellular and sub-cellular symptoms resulted from intoxication as well as locate symptoms of cellular death and reveal reactions in response to chronic and sub-lethal exposure in cells and tissues (Fontanetti et al., 2010; Kammenga et al., 2000).

Studies show that one of the main contaminants of the soil, metals, are selectively concentrated in only one or few organs, or in specific regions of the tissues in most of soil invertebrates and typically these organs are part of the digestive tract (Dallinger, 1993). For example, in millipeds (Köhler & Alberti, 1992), isopods (Dallinger & Prosi 1988) and springtails (Pawert et al., 1996), the epithelium of the midgut is the main target of metals. Thus, the epithelium of the digestive tract represents the first barrier against the intoxication of the whole organism (Walker, 1976).

In diplopods, some studies with this approach were performed using the digestive tube and the fat body (Hopkin et al., 1985; Köhler & Triebskorn, 1998; Triebskorn et al., 1999). Morphological alterations observed in the midgut (figure 4) and in the perivisceral fat body (figure 5) of the diplopod *R. padbergi* were successfully used as sublethal biomarkers in the evaluation of soils contaminated with complex substances such as sewage sludge (Godoy & Fontanetti, 2010; Nogarol & Fontanetti, 2010, 2011; Perez & Fontanetti, 2011a) and landfarming (Souza & Fontanetti, 2011; Souza et al., 2011).

In the studies performed with the diplopod *R. padbergi*, it was possible to observe tissular and cellular responses related to detoxification mechanisms such as increased cytoplasmic granules (spherocrystals) and intense release of secretory vesicles into the intestinal lumen of these invertebrates (Nogarol & Fontanetti, 2010; Perez & Fontanetti, 2011a). These secretory vesicles of the apocrine type seems to help in the detoxification of toxic substances initially absorbed by the organism and form a protector layer that would reduce the contact between the toxic agent and the intestinal epithelium.

The formation of agglomerates of haemocytes through the cells of the "fat body" layer was also observed and this response is directly related to a defence mechanism of the animal. According to van de Braak (2002), haemocytes can migrate to the injury site in the tissue by a chemotaxis process that results in inflammation. By this inflammatory reaction, these cells act in the removal of toxins and possibly help in the re-absorption of the damaged epithelium in order to maintain the homeostasis of the organism. In a recent review conducted by Perez and Fontanetti (2011b) it becomes clear that this tissular response is common in different invertebrates exposed to environmental stress conditions. According to the authors, the monitoring of the number of haemocytes can be used as a measure of stress in sentinel species due to environmental contamination.

The mechanisms of defence and detoxification require high and continuous energy expenditure, especially when the organism is exposed to a toxic agent for a long period. In this sense, histological and ultrastructural studies showed some of the main responses of this invertebrate related to higher energetic needs. Nogarol and Fontanetti (2011) observed at an ultrastructural level a high increase in the number of tracheioles between the cells of the "fat body" layer that compose the midgut of diplopods sub-chronically exposed to sewage sludge. The authors suggest that a higher oxygenation of the tissue was necessary to enable the formation of molecules of adenosine triphosphate (ATP), used in the detoxification mechanisms.

Toxic agents may be able to cause cellular death by necrosis, evidenced mainly by the intense cytoplasmic vacuolization in the principal cells of the midgut epithelium of diplopods exposed to landfarming (Souza & Fontanetti, 2011) and sewage sludge (Nogarol & Fontanetti, 2011; Perez & Fontanetti, 2011a). In addition to the cytoplasm, other cellular

compartments were affected by the exposure to toxic agents leading to cellular inviability. In these cases, damaged cells are expelled towards intestinal lumen.
Samples of landfarming and sewage sludge presented genotoxic action, evidenced by the occurrence of nucleus fragmentation in the principal epithelial cells, karyolysis in the nucleus of the cells of the fat body layer (Souza & Fontanetti, 2011) and loss of integrity of the nuclear envelope of hepatic cells and cells of the "fat body" layer of the midgut (Nogarol & Fontanetti, 2011).

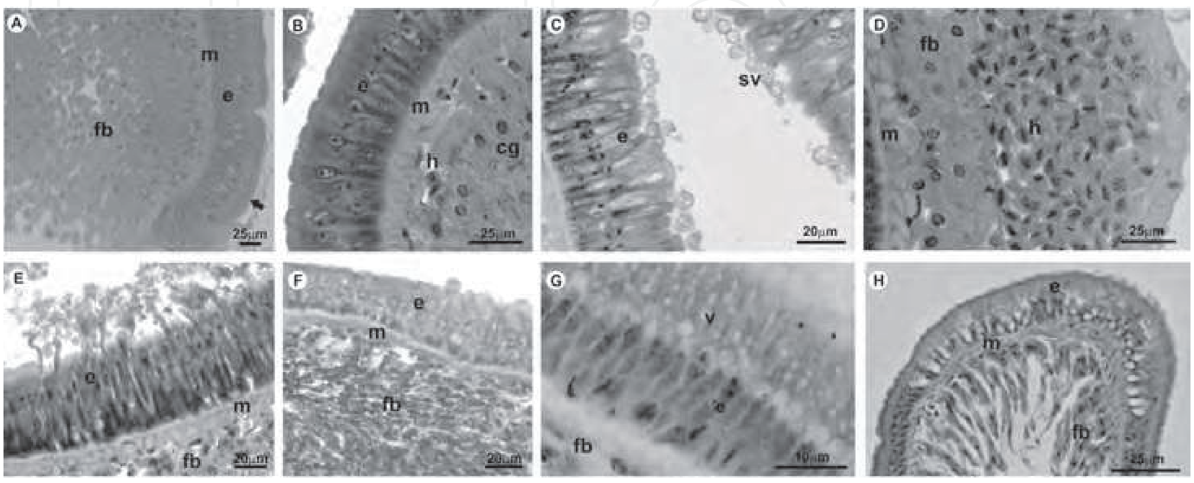

Fig. 4. Midgut of the diplopod *R. padbergi* stained with Hematoxylin-Eosin. Unexposed animals (A; B); Animals exposed to sewage sludge (C-H). secretion vesicles (C); haemocytes aglomeration (D); epithelium renewal (E); increase of cytoplasmatic granules in "fat body layer" (F); cytoplasmatic vacuolization (G); volume reduction of the cells in "fat body" layer of midgut (H). e=epithelium; m= muscle layer; fb= "fat body" layer; h= haemocytes; v= vacuole; sv= secretion vesicle; * dilatation of intercellular space (Photos: Larissa Rosa Nogarol; Raphael Bastão de Souza and Tatiana da Silva Souza)

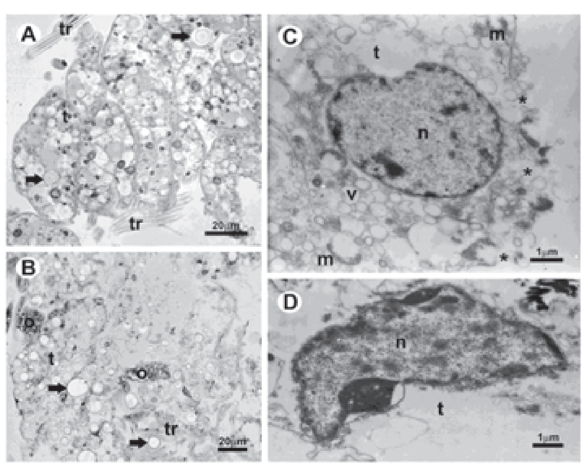

Fig. 5. Perivisceral fat body of the diplopod *R. padbergi* stained with Hematoxylin-Eosin (A; B) and submitted to TEM routine (C; D). Unexposed animal (A); Animal exposed to sewage sludge (B-D). Loss of cell limit and increase of spherocrystal (B); Cytoplasmatic vacuolization and loss of cell membrane integrity (C); Nucleus deformation (D). t= trophocyte; tr = tracheoles; o= oenocyte; m= mitochondria; n= nucleus; v= vacuole; arrows= spherocrystals; *= loss of cell membrane integrity. (Photos: Raphael Bastão de Souza and Larissa Rosa Nogarol)

In diplopods, the fat body, both parietal and perivisceral, is constituted of trophocytes and oenocytes (Fontanetti et al., 2004) and presents intense metabolic activity, such as storage of lipids, uric acid and proteins as well as storage, neutralization and excretion of substances that are not useful (Fontanetti et al., 2006; Hopkin & Head, 1992; Hubert, 1979). In this sense, Souza et al. (2011) exposed diplopods of the species *R. padbergi* in bioassays containing industrial soil contaminated by PAHs and metals (landfarming) in order to analyze histological and histochemical alterations in the perivisceral fat body. The authors concluded that the fat body can be used as a target organ and that the alterations observed, such as loss of integrity of the plasmatic membrane, cytoplasmic disorganization and depletion of energetic reserves can be considered stress biomarkers in this animal. Similar responses were observed in animals exposed to sewage sludge (Abe et al., 2010).

4.2 Genotoxicity biomarkers

The increase in the genotoxic load in the terrestrial ecosystems by the release of chemical products and physical agents can cause impact on the organisms, inducing increase in the frequency of mutations; such effects can lead to a decrease in the size of the population and, eventually, extinction of species and consequently affect the stability of this ecosystem (Majer et al., 2005). In this sense, it became necessary to develop different tests to evaluate the genotoxic potential of soil samples.

Due to the highly conserved structure of the genetic material, it is possible to use a wide variety of species in genotoxicity tests; currently, the most widespread methods for the routine tests are based on the use of indicator bacteria and also basidiomycetes fungi, plants, insects and cultured mammalian cells or even laboratory animals for mutagenicity tests.

According to the literature, the Ames test is the most widely used in genotoxicity evaluations of soils and leachate (Claxton et al., 2010; Wölz et al., 2011). This test, also known as *Salmonella*/microsome, consists, basically, in the employment of strains of the auxotrophic bacteria *Salmonella typhimurium*, i.e., deficient in the synthesis of the aminoacid histidine; the strains of these cells are unable to grow in minimum medium, where the mutagenic compounds are able to restore the synthesis capacity of this aminoacid, thus, the mutagenic expression corresponds to the growth of the colony in a minimum culture medium and it can be easily detected by counting the colonies (Umbuzeiro & Vargas, 2003). However, due to the low sensitivity of the Ames test for heavy metals, more studies should be directed to the development of bioassays with higher organisms (Gatehouse et al., 1990, as cited in Lah et al., 2008).

Meristematic cells of *A. cepa* and *V. faba*, for example, constitute an effective cytogenetic material to analyze chromosome aberrations (figure 6) caused by soil pollution. The use of meristematic cells makes possible the quantification of several morphological and cytogenetic parameters (endpoints), including the morphology and growth of roots and determination of several parameters of cytotoxicity, genotoxicity and mutagenicity. The analysis of the cytotoxicity can be done by determining the mitotic index and cell death. The induction of aberrant metaphases, anaphases and telophases, such as bridges, loss and chromosome stickiness, polyploidy, irregular nuclei and nuclear buds are parameters for the genotoxicity analysis, while the micronuclei and chromosome breaks allow the mutagenicity analysis (Fernandes et al., 2007; Leme & Marin-Morales, 2008; Souza et al., 2009).

Chromosome aberration test concerns the discovery of the mechanisms of action of a particular agent, since the division process is well known. Kovalchuk et al. (1998) state that the chromosome aberrations assay with *A. cepa*, can be used as a tool for quantifying and monitoring genetic alterations in soils radioactively contaminated. Moreover, the chromosome aberration assay in onion was the first of nine plant systems accepted in the Genotoxic Program of the Environmental Protection Agency (USEPA) and is widely used for monitoring residual water. Such sensitivity is attributed by Ma et al. (1995), to the large size of the chromosomes and because they are mostly metacentric. On the other hand, bioassays based on chromosome aberrations, in certain cases, tend to be replaced by less time-consuming techniques, such as the micronucleus assay (MN), which can be performed with mitotic cells in roots (of *V. faba* or *A. cepa*) or meiotic cells, in tetrads of *Tradescantia* (Misik et al., 2011).

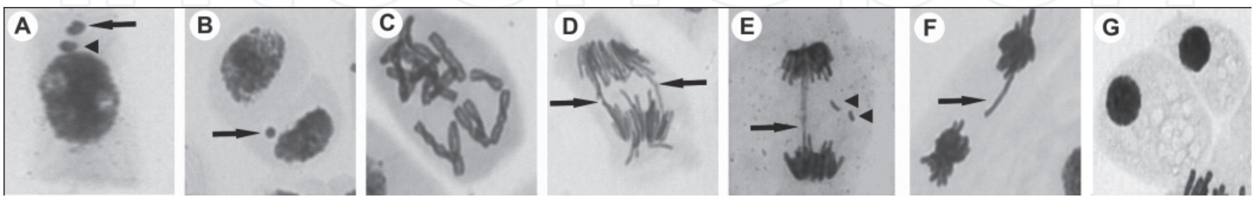

Fig. 6. Alterations observed in *A. cepa* meristematic cells. (A) nuclear bud (arrow head) and micronucleus (arrow); (B) micronucleus (arrow); (C) C-metaphase; (D) anaphase with chromossomal bridge (arrows); (E) telophase with cromossomal bridge (arrow) and chromossomal break (arrows head); (F) telophase with cromossomal delay (arrow); (G) cell death. (Photos: Cintya Aparecida Christofoletti)

The micronucleus test in *Tradescantia* (Trad-MCN) (figure 7) is a sensitive mutagenicity test, of short exposure and simple evaluation, applicable in the species *T. pallida* and in the clones BNL 4430 and KU 20 (Misik et al., 2011). Besides the Trad-MCN, it is possible to evaluate mutations in somatic cells of the staminal hair (Trad-SHM) in young inflorescences of the hybrid clone BNL 4430 (Brookhaven National Laboratory). However, currently, the clone KU 20 (Kyoto University) is more applicable to this technique due to the higher number of inflorescences per cycle. The mutation results in the expression of the recessive allele, which implies in the phenotype of pink colouration. The high rate of pink cells, as well as the loss of reproductive capacity are indicative of mutagenicity (Ma et al., 1996).

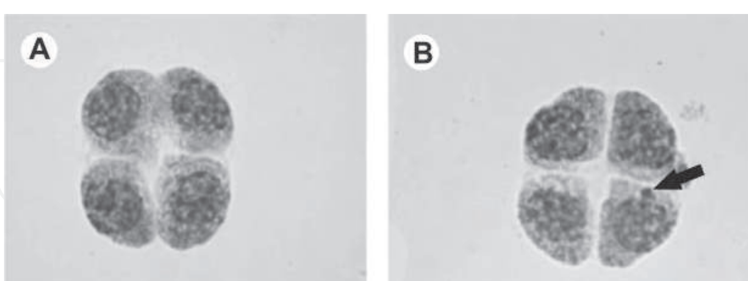

Fig. 7. Micronucleus in polen cells of *Tradescantia* (arrow in B). (Photos: Janaína Pedro-Escher)

4.3 Molecular biomarkers

The use of molecular biomarkers in the environment monitoring represents a significative tool for the evaluation of the contamination in different organisms. Despite morphological markers provide good qualitative evidence of damages caused by certain pollutants, it is known that biochemical alterations resulted from the toxic action of a contaminant are early evidence of negative effects of the exposure, since they precede the onset of visible damages.

Following, it is presented some biochemical biomarkers extensively used in studies of environmental impact, including ecotoxicological analyses of the soil.

4.3.1 Enzymatic antioxidants

The use of enzymatic activity as biomarker is due to the fact that toxic compounds have high affinity for electron pairs found in the aminoacids that form the enzymes (Cogo et al., 2009). One of the main monitored parameters in ecotoxicological analyses of the soil is the concentration of metals. Exposure to metals can intensify the production of reactive oxygen species (ROS), which are normally produced in non-stressed cells and their excess can lead to the oxidative stress and cause harmful effects (Barreiros et al., 2006).

When a cell undergoes oxidative damage, the injuries are minimized in the different organisms by enzymatic and non-enzymatic antioxidants (Freitas et al., 2008). Among the enzymatic antioxidants, some examples would be the superoxide dismutase, catalase, gluthathione reductase and gluthathione -S-transferase (Mishra et al., 2006).

Superoxide dismutase catalyzes the formation of H_2O_2 from O_2. This enzyme was the first discovered among the enzymatic antioxidants and, generally is one of the first to act against damages caused by ROS (Nordberg & Arnér, 2001).

Now, the catalase function is to facilitate the removal of H_2O_2, degrading it in H_2O and O_2. Thus, it reduces the risk of forming the radical hydroxyl from H_2O_2, since this oxygen reactive species is one of the most harmful to the biological systems (Betteridge, 2000; Diplock et al., 1998).

The metabolism of gluthathione is one of the main antioxidant defence mechanisms in the living systems (Valko et al., 2006) and specific metals can induce the synthesis of this compound in different species (Backor et al., 2007). In order to perform its function as oxidant agent, gluthathione must be in its reduced form, reaction catalyzed the enzyme gluathathione reductase (Creissen et al., 1994).

Another important defence system against the increase of free radicals involves the enzyme gluthathione peroxidase, which acts in the removal of hydrogen peroxide and lipid peroxides from the cell (Rover Junior et al., 2001). One of the forms of the gluthathione peroxidase is the gluthatione-S-transferase, one of the most studied detoxicant enzymes in different organisms, since it has an essential role in the cellular response to the stress caused by herbicides in plants. It is considered a detoxification enzyme because it metabolizes a great variety of xenobiotic compounds, catalyzing their conjugation with the reduced molecule of gluthathione and forming substances of low toxicity (Malmezat et al., 2000).

According to Almeida (2003), depending on the type of contaminant and exposure period of the organism to the contaminated environment, the activity of the antioxidant enzymes can be stimulated or inhibited. Generally, the increase of the enzyme activity results from an increase in the production of ROS, which leads to a exacerbated induction of enzymes; now, the decrease can be related to prolonged exposure of the organism to environments highly contaminated, where the production of ROS and the consequent deleterious effects of such production surpasses the defence efforts of the organism.

Several other studies describe alterations in the enzymatic activities of the superoxide dismutase, catalyse, gluthathione reductase and gluthathione peroxidise in different organisms exposed to stress conditions, especially metals, corroborating their use as effective biochemical biomarkers in the evaluation of environmental impacts (Bocchetti et al., 2008; Cogo et al., 2009).

4.3.2 Heat shock proteins

All organisms, from bacteria to mammals, respond to different environmental stress conditions by the synthesis of highly conserved proteins, known as heat shock proteins (HSPs) (Hamer et al., 2004). They are so called because they were first described in cells of *Drosophila melanogaster* during exposure to high temperatures. At the time, it was verified that the exposure of cells to heat produced a new pattern of thickening of chromosomes, which represented specific sites of transcription for the synthesis of proteins. The stress induced the expression of certain genes, which led the cell to produce a certain class of proteins, so-called heat shock proteins. Later, researchers observed that these proteins were expressed in almost all living beings, and not only in response to heat, but also when the cell was exposed to a series of other stressing factors (toxic concentrations of metals, organic pollutants, temperature, osmolarity, hypoxia/anoxia and ultraviolet radiation), and then they began to be called "stress proteins" or "anti-stress proteins" (Meyer & Silva, 1999).

Many toxicants affect the correct conformation and consequently, the function of different proteins. In this condition, where the proteins are found incorrectly folded inside the cell, it is initiated a stress response. The HSPs take action, acting as molecular chaperones, since they bind to other proteins, regulating their conformation, movement through the membrane or organelles and enzymatic activity (Calabrese et al., 2005). Therefore, they avoid incorrect interactions between proteins, helping in their synthesis, folding and degradation (Meyer & Silva, 1999).

According to Bierkens (2000), HSPs are one of the main cellular markers in the evaluation of the toxicity of different compounds and are widely used to monitor ecosystems. Such monitoring has shown high levels of HSPs in the tissues of invertebrates collected in contaminated areas, when compared to those animals existent in uncontaminated environments (Bierkens, 2000; Malaspina & Silva-Zacarin, 2006).

These proteins are found highly conserved in all the living organisms (Burdon, 1986) and can be classified according their molecular weight into four families: HSP90 (90 kDa), HSP70 (70 kDa), HSP60 (60 kDa) and small HSPs. The family HSP70 is one of the most studied and several studies have shown its induction in stress conditions by heavy metals (Köhler et al., 1992, 1996b; Nadeau et al., 2001; Zanger et al., 1996).

Monari et al. (2011) worked with the mollusc species *Chamelea gallina*, observing an increase in the expression of HSP70. The authors affirm that the induction of HSP70 can be considered an adaptation mechanism associated with changes in the environmental parameters.

Silva-Zacarin et al. (2006), using immune-histochemical methods, observed an increase in the levels of the products of the positive reaction to HSP70 in the salivary glands of bees treated with acaricides in comparison with the control group. Moreover, they also verified alterations in the immune-reactivity between the nucleus and cytoplasm according to the acaricide used and the treatment period. According to the authors, the determination and location of HSP70 by immune-histochemistry can be useful to detect cellular responses to chemical stressors.

Köhler et al. (1992) points out the HSP70 in invertebrates of soil as a possible tool in the monitoring of environmental toxicants. In a study conducted by Zanger et al. (1996), the authors exposed adult of the diplopod *Julus scandinavius* to substrates contaminated with different concentrations of cadmium and investigated the expression levels of HSP70. The analyses showed that an increase in the concentration of cadmium in the animals diet resulted in high levels of HSP70.

According to Zanger et al. (1996), the so-called hepatic cells of diplopods can present a strong expression of HSP70 in stress conditions induced by heavy metals. These cells are found disperse through the cells of the "fat body" layer that compose the midgut and one of their functions is to help in the detoxification of the organism (Hopkin & Read, 1992).

In a study carried out by Nadeau et al. (2001), it was investigated the feasibility of using HSP70 as marker of the presence of soil toxicants in the midgut of the earthworm *L. terrestris*. The authors concluded that HSP70 can be efficiently used in the assessment of the toxicity of soils using test organisms exposed under laboratory conditions.

5. Conclusion

Due to the constant release of harmful substances into the terrestrial environment, it is necessary to know their action on the organisms present there, in order to avoid triggering a possible unbalance in the ecosystems. In this sense, we tried to present potential bioindicators and biomarkers for ecotoxicological analyses of the soil. It is important to highlight that the choice of the bioindicator organism is essential to the success of environmental monitoring. Moreover, the combined use of morphological, biochemical and genotoxic methods in the evaluation of injuries in sentinel species is interesting since it provides a more complete understanding of the action of contaminants on organisms exposed, besides providing a greater reliability to the results obtained in the researches. We believe that understanding the importance of the combined use of different methodologies in assessing the toxicity of substrates will be highly beneficial for the future work of researchers in Ecotoxicology.

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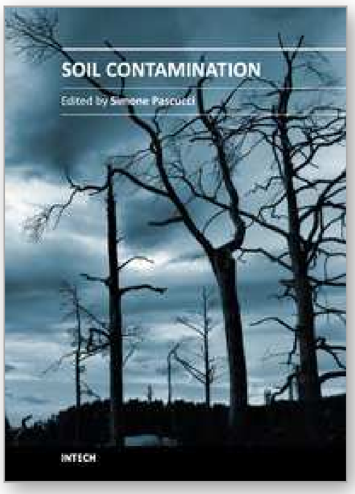

Soil Contamination

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Soil contamination has severely increased over the last decades, mainly due to petroleum hydrocarbons, solvents, pesticides, lead and other heavy metals from industrial wastes and human activities. The critical point regarding contaminated soil monitoring is the intrinsic difficulty in defining fixed monitoring variables and indicators as the establishment of any a priori criterion and threshold for soil quality can be still considered subjective. This book is organized into eight chapters and presents the state-of-the art and new research highlights in the context of contaminated soil monitoring and remediation strategies, including examples from South America, Europe and Asia. The chapters deal with the following topics: - monitoring of dioxin, furan, hydrocarbons and heavy metals level in soils - bioindicators and biomarkers for the assessment of soil toxicity - use of reflectance spectroscopy for soil contaminants and waste material detection - remediation technologies and strategies.