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# **Ecotoxicological Tests as a Tool to Assess the Quality of the Soil**

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Additional information is available at the end of the chapter

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## **Abstract**

Terrestrial ecotoxicology is used to evaluate the effects of substances that, whenever added to the soil, have impact on organisms and help measuring the responses from changes in the lethality, reproduction, development, and behavior of standardized soil organisms. Terrestrial ecotoxicology is a new tool that has been introduced in many countries, including Brazil, and yet little used. However, it is already widely used in Europe, besides being mandatory in research to indicate the toxicity of waste discharged in the soil. The aim of this chapter is to emphasize the importance and need of developing studies focused on the use of terrestrial ecotoxicology as a tool to assess fast and reliable responses from the toxicity of substances incorporated to the soil.

**Keywords:** contamination, bioindicators, biomarkers, pesticides, soil fauna

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## **1. Introduction**

The environment has been exposed to contamination caused by many toxic agents due to human action. Environmental impact, such as soil, surface water, and groundwater contamination, is mainly caused by deactivated industries, contaminant leakage, pesticide use, and inadequate solid and liquid waste disposal. Soil protection and the protection of its inherent communities are acknowledged as the primary aim of environmental policies worldwide [1].

Although the most obvious use of soil lies on plant cultivation for food production, it is also responsible for helping sustainability maintenance. Soil retains water, regulates water resources, and filters and conducts rain water to underground aquifers. It also plays the role

of recycling raw materials and nutrients, besides being the habitat of a wide variety of organisms such as bacteria, fungi, viruses, nematodes, insects, and worms, among others. The food consumed by living organisms is produced in the soil [2].

The soil, besides being the substance for most man-made food, holds a large amount of pollutants. Chemical and organic fertilizers, pesticides, and other materials applied to the soil often contribute to water and air pollution. Soil is a key component of environmental chemical cycles and an important part of natural resources on Earth. The quality of the soil and climate, which enables productivity, is the most valuable asset of the society [3, 4].

Ecotoxicological tests are internationally acknowledged as complementary tools to chemically analyze soil contamination. However, countries, such as Brazil, do not require the conduction of ecotoxicological tests with soil organisms to evaluate contamination, since it is only based on chemical indicators [5]. The behavior and toxicity of soil elements, or compounds, should not be only assessed based on chemical parameters. Actually, it should be of the utmost importance to include biological parameters in these investigations, since chemical analyses separately applied to the compounds may not show their behavior in the environment [6].

Terrestrial ecotoxicology seeks knowledge about the consequences of chemical-substance discharge in the environment on the organisms living in it. Therefore, it is essential understanding, to which extent, how hazardous the use of chemicals, alone or in mixtures, is, as well as where its effects are observed by monitoring the lethal, morphological, behavioral, physiological, cytogenetic, and biochemical effects on organisms exposed to these pollutants [6, 7].

Among many organisms in soil fauna, which is divided into macro-, meso-, and microfauna, one finds bacteria and fungi and species known as bioindicators. These organisms indicate environmental changes at their early stages and identify several modification types before these changes become worse, besides determining the pollution types capable of affecting a given ecosystem. They also have the ability to help monitoring and making more accurate and less impacting decisions on soil management. There are standardized bioindicator species around the world, such as *Collembola*, earthworms, nematodes, and enchytraeids. The use of bioindicators in monitoring programs helps detecting environmental changes at their early stages or the effectiveness of measures taken to improve environmental quality [7].

## 2. Soil quality

The quality of the soil is defined by “its ability to function within an ecosystem in a way that it sustains biological productivity, maintains environmental quality, and promotes plant and animal health” [8]. Overall, knowing the soil characteristics involved in the development and sustainability of ecosystems is a useful tool.

Soil quality is assessed through the characteristic of indicators that measure or reflect environmental status or sustainability conditions of ecosystems. Soil quality indicators can be classified as physical, chemical, and biological.

## 2.1. Physical indicators

The main physical indicators in place are texture, thickness (horizons), soil density, porosity, water, and structure.

Texture refers to particle proportions or to sand, silt, and clay fractions in the soil mass. It is closely related to water retention and transport, soil structure, nutrient content, and organic matter, besides its strong influence on soil erosion processes [9, 10].

Thickness, mainly that of the surface horizon, is essential, since it is the place where the greatest biota activity takes place. Consequently, the surface horizon is a suitable place for organic matter and nutrient cycling. Soil depth plays a key role given its water storage ability and influence on nutrient supply for plants [10].

The diversity of mineral and organic components, as well as their proportion, determines the density of soil materials. This density changes depending on texture and on soil structural conditions [9, 11].

Porosity is determined by porous space. Part of the soil after the arrangement of solid components is occupied by water and air under natural conditions. Sand retains little water, because its large porous space allows free water drainage from the soil. Clay adsorbs relatively large amounts of water, and its smaller porous space retains water, even with gravity forces [11, 12].

Water amount in the soil depends on climatic factors and on soil texture, structure, and porosity. Water retention capacity reveals the amount of water the soil can store. Hydraulic conductivity is defined by the speed water reaches when it moves in the soil and by its infiltration rates [11, 12].

Soil structure is determined by the geometric arrangement of primary (sand, silt, and clay) and secondary particles (aggregates maintained by cementing agents such as iron, silica, and organic matter) [13].

## 2.2. Chemical indicators

Many soil functions such as nutrient reservoir, filtration of substances dissolved in water, chemical reaction accelerator, and pollutant immobilizer would not be possible without the presence of chemical components. Organic matter (OM), cation exchange capacity (CEC), hydrogen ionic potential (pH), and chemical elements are often used as chemical indicators of the quality of the soil [14].

Organic matter (OM) is the set of all organic materials in the soil. It is correlated to most soil properties, besides being a key indicator of the quality of the soil [15].

CEC stands out as one of the basic functions of the soil that lies on providing nutrients to plants. This indicator is found on the surface of solid particles in the soil due to electrical charges (Al, Ca, K, Mg, Mn, and Na), although it can be changed by pH and OM [14, 15].

pH is the indicator used to measure soil acidity, and changes in it can affect nutrient availability, microbial populations, and the availability of chemical elements in the soil.

Chemical elements play many roles, but it is possible roughly framing them in nutrients or toxic elements.

Soil nutrients can be divided into two groups:

- Macronutrients (Ca, K, Mg, N, P, S), which are mostly needed by plants.
- Toxic elements, which are considered heavy metals.

Have in mind that the same chemical element, in a certain concentration, can be a nutrient but toxic in another one.

### 2.3. Biological indicators

Biological indicators, such as microbial biomass, mineralizable nitrogen, microbial respiration, enzyme activity, and metabolic quotient, are fundamental for nutrient cycling and for estimates on soil ability to influence plant growth. In addition, microorganisms provide rapid responses to changes in the environment due to the abundance of metabolic and biochemical activities; therefore, they have great potential to be used as a tool to assess the quality of the soil [16].

The microbial biomass is represented by living components in soil organic matter [17]. This variable controls functions such as the decomposition and accumulation of organic matter or transformations involving mineral nutrients. Biomass provides information about changes in the organic properties of the soil, about changes caused by crops or by vegetation removal, about regeneration after topsoil removal, and about the effects from pollutants such as heavy metals and pesticides [16, 17].

The nitrogen (N) in the soil is the organic compound indirectly available to plants. The N content conversion from its organic form to its mineral one (mineralization) is mainly carried out by bacteria belonging to genus *Rhizobium*, which convert atmospheric nitrogen ( $N_2$ ) into ammonia ( $NH_3$ ) through an enzymatic complex. From this point on, ammonia can be incorporated to different forms of organic nitrogen available for plants. From a soil viewpoint, this potential organic N conversion into mineral N (potentially mineralizable nitrogen) has been considered an important factor; therefore, it is an indicator recommended to measure the quality of the soil [8].

Soil respiration reflects the microbial activity and is defined as the biological oxidation of organic matter into carbon dioxide ( $CO_2$ ), which is conducted by aerobic microorganisms [18]. This indicator occupies a key position in carbon cycles observed in terrestrial ecosystems due to oxidation [19].

Enzymes rule the biological catabolism of organic and mineral components in the soil. This process is closely related to organic matter, physical properties, and microbial activity and biomass; therefore, it is widely used to assess the quality of the soil, since it indicates changes in microbial activity and the presence of pollutants in the soil [14, 20, 21].

The respiratory coefficient represents the relation between carbon in the microbial biomass and total organic carbon (TOC). This coefficient indicates microbial biomass efficiency in using the available carbon for biosynthesis [21, 22].

Besides the aforementioned biological indicators, soil fauna can be used as quality indicator. The biological diversity of the soil is fundamental to maintain soil productive ability; therefore, it is of great importance for the decomposition and mineralization of organic residues, since it favors nutrient availability for plants and even for other individuals. In addition, the soil fauna is sensitive to environmental changes, biological, physical, or chemical. Earthworms (*Oligochaeta*) and isopods (*Isopoda*) are among the organisms most studied as indicators [23].

### 3. Contamination of soils

Soil contamination is one of the main environmental issues worldwide [1]. The unconscious use of the soil for agricultural activities, for the disposal of waste, chemicals, and industrial waste has been the cause of concern for centuries. Besides huge damages to the environment, a large amount of contaminated soils unviable for agriculture or for construction sites is the consequence of such inappropriate discharge [24]. Agricultural production has led to the increased use of pesticides to control pests and weeds. In addition to active toxic ingredients, many agricultural products contain potentially polluting elements or compounds, such as trace elements and emulsifying surfactants, among others [25, 26]. There has been simultaneous increase in the application of sewage sludge, industrial waste—composed of urban waste—and of agricultural waste for disposal or recycling purposes. These residues have high contents of organic matter and mineral elements capable of improving the chemical, physical, and biological properties of the soil. However, these residues may contain trace elements, pathogens, and many other substances that cause environmental damages [26, 27].

The term “soil contamination” refers to the presence of toxic substances belonging to chemical classes such as inorganic ions (metals), organic solvents, radioactive substances, pharmaceuticals, polycyclic aromatic hydrocarbons (PAHs), and pesticides (herbicide, insecticide, and fungicide) [28].

According to the International Union of Pure and Applied Chemistry (IUPAC), inorganic pollutants are described as “toxic elements.” They comprise metal and metalloid elements formerly called “heavy metals.” This nomenclature includes elements that, at low concentration, are biologically essential to living organisms. However, when these essential elements are observed at concentrations higher than the required ones or above the recommended limits, they also cause imbalance and exhibit some toxicity.

The displacement of water, soil, and air contaminants, as well as the interface between different compartments, is determined by processes related to the chemical properties of substances in the soil and to environmental compartments. Soil is one of the most complex matrices in the environment due to its heterogeneity. Different soil types present a wide variety of particle sizes and ecophysiological characteristics [14].

Contaminants that reach the lithosphere move by diffusion, that is, water moves through gaps between soil particles. Contaminant displacement speed depends on molecular weight and on the concentration of the contaminant gradient, as well as on soil characteristics such as humidity, clay type, specific area, cation exchange capacity, pH, redox potential, temperature,



porosity, and permeability. Soil contamination presents intrinsic characteristics such as cumulative characters and low mobility of pollutants. However, this mobility becomes greater in soil recording relatively low pH or large amounts of sand to the detriment of clay, since these factors make the solubility of toxic elements easier in the environment. Consequently, the soil becomes susceptible to water leach to underground sheets and to other water bodies. Therefore, interactions among the chemical structure of contaminants, soil properties, and entry mode in the environment determine whether a specific substance is persistent and whether it is potentially hazardous to soil compartment [12–15].

The soil must be understood as a living being due to its characteristics, since its biota plays a fundamental role in physical-chemical equilibrium. The soil gets polluted by negative changes in the existing equilibria, which lead to conditions that impair, or makes unfeasible, the life in the assessed area. They also cause environmental damages that can take millennia to be fixed.

## 4. Terrestrial ecotoxicology

Terrestrial ecotoxicology aims at evaluating the effect of toxic substances on organisms representative of terrestrial ecosystems. This area involves the transportation, distribution, transformation, and final destination given to contaminants found in the terrestrial environment.

Biological processes may be more sensitive to soil changes based on physical and chemical properties, since they suggest that biological indicators could potentially provide early warnings about risks posed to ecosystems [7].

The soil is a heterogeneous mixture of biotic and abiotic factors, besides being inhabited by a wide community of organisms. The fundamental functions of the system depend on its structural and functional integrity. This functionality faces direct impact from changes, and it requires many parameters in order to accomplish a better evaluation [4, 8, 10].

Terrestrial ecotoxicology uses ecotoxicological tests—internationally standardized by the International Organization for Standardization (ISO) and by the Organization for Economic Cooperation and Development (OECD)—which expose bioindicators (animals or plants) to soils contaminated with products or residues of interest in order to qualify and quantify the aforementioned negative effects. Different contamination levels are used in studies in this field [5, 29, 30]; therefore, Inferring the toxicity of the assessed substance and contributing to establish the limits of its use or disposal is possible.

Invertebrate populations in the soil are an appropriate tool to assess the degree of soil disturbance or land use intensification [5] due to human activities [30].

### 4.1. Ecotoxicological tests

Ecotoxicology is applied through tests that measure the toxicity of substances found in the environment by exposing standard living organisms to them [31].

Ecotoxicological tests allow assessing environmental contamination caused by different pollution sources. These tests have the advantage of covering a wide variety of biological

substances available in a sample. They have the inherent ability to detect deleterious effects produced by a toxic agent, or mixture, on living organisms. Thus, these tests allow evaluating how hazardous these substances are.

A large number of ecotoxicological trials have been developed, or improved, due to a wide variety of studied species and ecosystems. Aquatic, sedimentary, and terrestrial systems can be used in tests to verify the degree of contaminations caused by toxic agents and their possible ecological implications [32, 33].

#### *4.1.1. Avoidance test*

Among the ecotoxicological tests available, the behavioral test (avoidance test) is a rapid method applied to determine the bioavailability of chemicals or the contaminants in the soil. Although this test is a simple and rapid assay, it has high ecological significance, since avoidance indicates site rejection and population decrease caused by stressor agents such as contamination [34].

The behavioral response results from the level of the organism, which can be defined as the action, reaction, or activation of a given system. This system is subjected to a set of specific conditions that represent the integration of biochemical and physiological processes [35].

Avoidance tests also substantiate the application of other ecotoxicological tests (acute and chronic), since they indicate whether a substance is influencing the physiological and metabolic functions of test organisms.

#### *4.1.2. Acute toxicity test*

Acute toxicity tests (mortality test) assess contamination after short-term exposure to high contaminant doses (from 24 h to 14 days). Overall, acute toxicity test results showed that lethality is the main effect of contamination; however, other manifestations such as decreased mobility can be observed [36].

Acute toxicity tests are relatively simple, are inexpensive, and can be applied to a wide variety of organisms. However, they have the disadvantages of not indicating the contaminants responsible for the observed toxicity and the effects of contamination on the dynamics of assessed populations [37].

Results of acute toxicity tests include LC50/LD50 values (concentration/dose causing mortality to 50% of organisms tested) or EC50 (effective concentration or concentration causing an effect, other than mortality, that is, immobility to exposed organisms) [38].

#### *4.1.3. Chronic toxicity test*

Chronic toxicity (reproduction test) tests are closely linked to results of acute toxicity tests, since sublethal concentrations are calculated based on LC50. These tests evaluate the effects of lower contaminant concentrations for long exposure time. The observed effects are sublethal and emerge when the toxic agent concentration the organisms are exposed to allows the survival of these organisms. However, these concentrations affect one, or more, biological function of these organisms, and it influences reproduction and egg development and growth [40].



Chronic toxicity tests may be long and laborious; sometimes they range from 4 to 7 weeks, and these factors are the main disadvantage of applying this test type [37]. However, they are the most sensitive tests, which are considered the most relevant to predict the impacts on ecosystems, since they demonstrate the dynamics of populations over time [41].

Results of chronic toxicity tests can be expressed in CENO (concentration of observed effect, the highest concentration of the tested sample, which does not cause deleterious effect) or in CEO (concentration of observed effect, the effect of the lowest concentration on the body) [39].

The use of ecotoxicological tests aims at integrating different information to plan, to make decisions about public health, to take environmental control measures, to define remediation techniques, and to hierarchize the areas to be prioritized by environmental recovery programs.

#### **4.2. Biomarkers and bioindicators**

Biomarkers are tools used to measure the effects of the exposure to toxic compounds and its potential impact on living organisms, including humans. Bioindicators are changes resulting from the presence of xenobiotics in components and in cell biochemical processes, structures, or functions measurable in a system or sample [42].

Indicator organisms are used in the testes due to their characteristics, since they present very short ecological tolerance limit. Therefore, they present some physiological, morphological, and/or behavioral changes when they are exposed to certain contaminants [32].

The low cost of bioindicators is their advantage in comparison to conventional methods used to evaluate environmental quality. They can also be used in the cumulative evaluation of events observed within a given period of time to recover an environmental history that cannot be detected through other methods [23, 31, 32].

The soil hosts the greatest diversity of organisms on the planet; however, these organisms can be affected by the substances deposited in it [16, 34]. Hundreds of thousands of invertebrate species contribute to decomposition processes by crushing organic matter, improving the mineralization process and, consequently, the nutrient cycling and energy flow in ecosystems [16, 43].

Ecotoxicological tests have been widely used to evaluate the environmental impact of several pollution sources such as hydrocarbons [34, 36], toxic sludge from the ceramic [44] and textile industries [45], domestic effluent [46, 47], persistent organic pollutants (POPs) [48], and bovine manure [49].

However, ecotoxicological studies conducted in terrestrial environments remain relatively incipient in comparison to the ones involving aquatic environments. Most studies in this field are concentrated in Northern countries, and this evidence highlights the need of verifying the possible impacts of this situation on the soil of different regions of the world [27, 30].

Edaphic organisms are among the indicators used to measure the quality of the soil, given their importance to the decomposition, cycling, and regularization of nutrients in biological systems [23].

Earthworms (*Oligochaeta*) are among the most studied edaphic organisms to be used as bioindicators. These organisms are bigger than 2 mm (soil macrofauna) and have direct influence on soil functioning [50].

#### 4.3. *Eisenia* sp. (*Oligochaeta*, *Lumbricidae*)

Species belonging to genus *Eisenia* sp. (*Oligochaeta*, *Lumbricidae*), commonly known as Californian or red worms of California [16, 23], stand out among terrestrial fauna species used in toxicity tests. *Eisenia fetida* and *Eisenia andrei* naturally live in the topsoil, animal manure, and compost materials. They are soil organisms that participate in soil aggregation processes and in the decomposition of plant residues, thus maintaining soil fertility and the quality of agricultural and natural ecosystems [40].

Earthworms are exposed to contaminants through skin absorption and intake. A yellow fetid substance acts in defense of the earthworms when they are threatened by pores in the upper surface of the body—this substance keeps predators away from them [51–53].

*Oligochaetes* are often used to evaluate soil contamination because they ingest a large amount of soil and demonstrate the ability of accumulating pollutants [54–56]. They crush organic matter and produce excellent-quality humus, fact that facilitates water and air entry into the soil and helps combating erosion and recovering degraded soils [57].

According to [58], earthworms are widely used given their suitability for the bioavailability evaluation of many soil chemicals due to the following factors:

- Earthworms live on the ground and are in constant contact with the soil.
- They live in contaminated sites, and it allows the validation of chemical availability in the field.
- They are found in a wide variety of soil horizons.
- The epidermis of the worms is vascularized, but it has no cuticle, and it allows absorbing the contaminants straight from the soil.
- Earthworms ingest specific fractions of soil, and it provides the means for contaminant uptake.
- They present high body mass, and this factor helps determining the concentration of contaminants in the assessed individuals.
- Their physiology and the metabolism of metals in their organisms are well known.
- Availability of standard test protocols.
- Some species, such as *E. fetida*, are easy to cultivate, can be kept in laboratory under controlled conditions, and are tolerant to different soil types.

Pollutants in soils have direct contact with clay and with organic materials highly capable of binding to chemical compounds and substances [14, 15, 19]. Earthworms get in contact with pollutants when they excavate and ingest contaminated soil or litter; they also absorb

contaminants from the soil solution that passes through the cuticle. So earthworms can poison themselves with these pollutants; they can die or survive by incorporating or even bioaccumulating these pollutants in their tissues. This ability can be a threat to their predators, since earthworms are an important link in the terrestrial trophic chain; they are on the diet of several animal species [59–63].

Procedures followed in ecotoxicological tests conducted with earthworms are established by national and international standards. There are internationally acknowledged standards proposed by International Organization for Standardization (ISO) and Organization for Economic Cooperation and Development (OECD). However, some countries have their own standards. For example, in Brazil, the NBR 15537 (2014) and NBR ISO 17512-1 (2012) regulate the application of test with earthworm conducted to evaluate acute toxicity and behavior, respectively.

**Table 1** shows a selection of standardized toxicity test protocols developed by acknowledged institutions involving worms currently available for researchers.

Many studies focus on artificially contaminated soil; they use to add a single substance to the assessed soil. In addition, these studies are conducted under conditions that do not properly reflect the reality in the field.

4.3.1. Ecotoxicological studies already carried out with earthworms belonging to genus *Eisenia* sp.

**Table 2** shows a summary of results recorded by some authors in studies conducted with *Eisenia* sp., based on behavioral tests (avoidance).

**Table 3** summarizes the results recorded by some authors in studies with *Eisenia* sp. based on ecotoxicological trials conducted to measure lethality (acute test) and reproduction (chronic test).

Laboratory soil toxicity tests have advanced in recent years given the introduction of soil invertebrates in them; consequently, the adoption of avoidance toxicity tests has increased [34].

Test	Duration (days)	Species	Standard
Lethality	14	<i>E. fetida</i> / <i>E. andrei</i>	OECD 2017 (1984) ISO 11268 (2012)
Reproduction	28 + 28 <sup>1</sup>	<i>E. fetida</i> / <i>E. andrei</i>	ISO 11268-2 (2012) OECD 222 (2004)
Avoidance	2	<i>E. fetida</i> / <i>E. andrei</i>	ISO 17512-1 (2012)
Bioaccumulation	Until 21 + 21 <sup>2</sup>	<i>E. fetida</i> / <i>E. andrei</i>	OECD 317 (2010)
	7 to 28	<i>E. fetida</i>	ASTM E 1676-12 (2010)

<sup>1</sup>28 days for cocoon production + 28 days for juvenile hatching.

<sup>2</sup>First stage, up to 21 days for the absorption of the test substance; second stage, 21 days for elimination.

**Table 1.** Standardized toxicity tests using earthworms as test organisms.

Author	Results
[37]	They exposed <i>E. andrei</i> specimens to different substances (copper sulfate, pesticides, dimethoate, carbendazim, benomyl) in soil collected in a deactivated mine Results: avoidance—copper sulfate, 320 mg/kg, earthworms in the assessed mine showed no preference between the contaminated soil and the control
[36]	They evaluated the behavior of <i>E. fetida</i> specimens in samples contaminated with hydrocarbons Results: 96% of individuals fled the section containing the contaminated sample
[64]	They evaluated the behavior of <i>E. andrei</i> specimens exposed to three sludge types: treated domestic effluent, treated canning industry effluent, and the sludge from a galvanic treatment plant Results: Organisms presented greater attraction to sludge from the sewage treatment plant and treated canning industry effluent due to the higher organic matter concentrations in them. There was avoidance reaction at low concentrations of sludge from the galvanic industry, mainly due to the high content of chromium in it
[65]	The authors analyzed soil and sludge mixtures from three sewage treatment plants in Germany. Results: The sludge was toxic to the test organisms ( <i>E. fetida</i> ) at concentration 9 g sludge/kg soil (EC50, 13.4 g sludge/kg soil). About 100% contaminated soil evasion was observed at 45 g/kg
[66]	Assessed the effects of herbicides Diuron and fluazifop-p-butyl on <i>E. andrei</i> Results: The avoidance behavior in the soil evidenced that both herbicides caused significant avoidance response
[67]	Studied the avoidance behavior to silver forms (nanomaterials (NMs)) at four time points (24, 48, 72, and 96 h) Results: AgNO <sub>3</sub> and NM300K induced avoidance response in <i>E. fetida</i> at concentrations 10 and 100 times lower than the concentrations required for AgNM-PVP coated and AgNM-non-coated nanomaterials
[34]	The authors investigated the toxicity of a binary petroleum hydrocarbon (PHC) mixture to the avoidance response of five soil invertebrate species ( <i>E. fetida</i> , <i>Enchytraeus crypticus</i> , <i>Folsomia candida</i> , <i>Oppia nitens</i> , and <i>Hypoaspis aculeifer</i> ) Results: The avoidance of invertebrates in PHC-contaminated soil was similar in growth measurements applied to plant species sensitive to PHC-contaminated soil

**Table 2.** List of results recorded by some authors in a study focused behavioral tests conducted with earthworms.

There is lack of data about the effects of herbicides on earthworms because they are often seen as low or nontoxic. [40] investigated whether the widely used commercial formulations of glyphosate (GLF), tembotrione (TBT), and nicosulfuron (NCS), which are applied at three environmentally relevant concentrations, have adverse effects on different biomarkers and on the reproduction of the epigeic earthworm species *Dendrobaena veneta*. The tested herbicides did not have significant effect on reproduction success. GLF induced the acetylcholinesterase (AChE) activity after 7 days and NCS, after 28 days, whereas TBT caused up to 47% inhibition after 7 days. Only TBT caused significant change in catalase (CAT) after 7 days of exposure. Malondialdehyde concentrations (MDA) increased after NCS exposure (at any exposure period), but it only happened in GLF and in TBT after 7 and 28 days, respectively. The activity of measured biomarkers changed depending on the applied herbicide and on exposure time; it also suggested that oxidative stress plays an important role in the toxicity of the tested herbicides.

Author	Results
[63]	<p>Eight soil samples were collected from seven abandoned mines in the United Kingdom</p> <p>Results: Cocoon production and hatching rates showed that they were more sensitive to survival conditions or to weight changes. The most toxic soil presented low organic carbon, and sandy soil presented intermediate metal concentrations (7150–13,100 mg Pb/kg, 2970–53,400 mg Zn/kg)</p>
[64]	<p>Evaluated the behavior of <i>Eisenia andrei</i> specimens exposed to three sludge types: treated sewage, treated effluent from an olive plant, and sludge from a galvanic treatment plant</p> <p>Results: Mixtures containing domestic sewage and sludge from the olive industry increased the production of juvenile organisms and stimulated the growth of earthworms. However, the mixture with sludge from the galvanic industry caused significant decrease in the production of juveniles</p>
[68]	<p>The authors carried out an ecotoxicological evaluation with oligochaetes (<i>E. andrei</i>) living in soil treated with sewage</p> <p>Results: The applied doses did not present significant mortality under acute exposure, but they caused lethal effects under chronic exposure. In addition, the test organisms bioaccumulated metals (Cu, Hg, Pb, Zn) because they are bioavailable and due to the role played by Cu and Zn in the metabolism and physiology of oligochaetes</p>
[48]	<p>Evaluated the ability of <i>E. andrei</i> species in bioaccumulate hexachlorobenzene, which is a persistent organic pollutant (POP)</p> <p>Results: Annelids accumulated hexachlorobenzene in their tissues, and this process may result in biomagnification along food chains</p>
[49]	<p>Evaluated the toxicity of bovine clearance residues on the survival rate of <i>E. fetida</i></p> <p>Results: They found 100% lethality in individuals subjected to the treatment with waste at concentrations 84.12 mg/kg Zn and 323.11 mg/kg Cu.</p> <p>The increased Cu and Zn concentrations reduced the number of cocoons</p>
[47]	<p>The authors carried out acute and chronic toxicity tests with <i>E. fetida</i> specimens in sanitary effluent sludge.</p> <p>Results: The sewage sludge showed no acute toxicity effect. The reproduction test presented the deleterious effect of it on the reproduction of worms, since there was the absence of cocoons and of young organisms after the 14th day of testing</p>
[69]	<p>Evaluated dredged sediments in Guabanara Bay, Rio de Janeiro, Brazil, through toxicity tests. The bay has been highly impacted by the disposal of liquid domestic and industrial waste</p> <p>Results: <i>E. andrei</i> reproduction was sensitive to dilute sediment samples presenting EC 20 = 1.26%, EC 50 = 2.94%. In addition, surviving earthworms showed visible morphological damage in their epidermis</p>
[29]	<p>The toxicity of five pesticides (trichlorfon, dimethoate, carbendazim, tebuconazole, and prochloraz) typically used in rice farming was evaluated through the mortality and body weight of <i>E. fetida</i> specimens 7 and 14 days after the beginning of the experiment.</p> <p>Results: The insecticide dimethoate showed moderate acute toxicity, whereas the other tested pesticides showed low toxicity potential. However, weight loss was identified as a sensitive endpoint with the use of these pesticides</p>
[70]	<p>Evaluated the chronic toxicity of the abovementioned six neonicotinoids (NEOs) to <i>E. fetida</i>—its cocoon production, hatchability, cocoon weight, and adult weight were affected in the test</p> <p>Results: Cocoon production and hatchability were more sensitive than cocoon weight and adult weight. The reproduction of earthworms was significantly reduced at 56 days half-maximal effective hatchability concentrations (EC50) 0.37, 0.74, 1.30, 3.57, 1.20, and 0.70 mg/kg (acetamiprid, dinotefuran, clothianidin, thiacloprid, nitenpyram, imidacloprid), respectively. Most tested NEOs were highly toxic to <i>E. fetida</i></p>

**Table 3.** List of results recorded by some authors in a study focused on lethality and reproduction conducted with earthworms.



Ref. [68] evaluated bioaccumulation in organisms of oligochaetes exposed to soil treated with sewage sludge and found that earthworms absorbed Zn and Cu metals in their tissues. In addition, the chronic assay caused lethal effects, as well as the absence of cocoons, at the end of the bioaccumulation stage. This outcome evidences that the toxics found in sewage sludge were able to affect the reproduction of oligochaetes.

There was significant copper (Cu) uptake increase by earthworm (*Eisenia fetida*) when there was combined benzotriazole (BTR) pollution in the soil [26]. According these authors, water and soil environments contaminated with triazole can form complexes of metal ions and, therefore, affect the bioavailability and toxicity of some heavy metals.

Ref. [71] verified the toxicity rates in *Eisenia andrei* species exposed to different aluminum concentrations and found that the metal was toxic (457 mg Al/kg) at lower pH values (3.24). The growth and production of cocoons reduced at this pH.

Ref. [72] evaluated the toxicity of different aluminum concentrations to *E. fetida*. Their results indicated that its survival was not affected until 1444 mg Al/kg, but it reduced by 85% at concentration 2222 mg Al/kg.

Some factors influence test results, and they should be carefully evaluated based on their interference.

According to [73], soil characteristics in extremely sandy soils influence the results of ecotoxicological tests. Earthworms tolerate a wide range of pH (from 4 to 9), but they prefer neutral to slightly acidic pH conditions (from 5 to 7). Ref. [74] studied the reproduction of *E. andrei* in artificial soil and found reduced number of juveniles at pH values 4, 6.5, 7.5, and 8, as well as optimum cocoon production at pH between 5 and 6.

Earthworms prefer soil with high levels of organic matter. According to studies, earthworms strongly tend to avoid soil with low organic matter content [75]. In addition, several authors [76–78] indicate that organic matter forms stable complexes with metals by reducing their bioavailability and geochemical mobility.

Ref. [64, 69] point out that oligochaetes recognize the organic matter in domestic sewage and in sediments as real food sources.

Ref. [71] sought to analyze the reproduction of earthworms and the production of cocoons of organisms exposed to different Al concentrations and pH. Based on the recorded results, low Al concentrations and higher pH values reduced the production of cocoons. These authors suggest that increased oligochaete biomass may have implications in the reproduction of these animals, since organisms use much of their metabolism energy to increase their biomass; thus they reduce their reproduction and cocoon production rates.

Ref. [69] found biomass loss in test organisms subjected to the treatment with 20% dredged sediment from a pond receptor of domestic and industrial effluent. Weight loss likely resulted from the fact that energy reserves are mobilized to allow detoxification processes that reduce the energy allocated for growth purposes.



The greater attraction of organisms to soil with high organic matter content does not exclude toxicity likelihood, and this observation evidences the need of carrying out a test at chronic level.

Thus, it is essential knowing soil characteristics, since they can influence stress factors in organisms, other than the ones related to the contaminant that influence test results.

Recently, lethality and reproduction are not only analyzed through toxicity trials. Biochemical responses and DNA damage are complementary approaches to standard toxicity tests, since they provide more information about body responses to stress in mixtures [79].

Ref. [79] evaluated the activity of superoxide dismutase, acetylcholinesterase, cellulase, and DNA damages in *E. fetida* living in soil contaminated with heavy metals. Based on their results, there was lower sensitivity to superoxide dismutase enzyme activity, whereas dismutase, acetylcholinesterase, and DNA damage were more sensitive.

Ref. [80] evaluated changes in superoxide dismutase, catalase, peroxidase, cellulase, and malondialdehyde in *E. fetida* exposed to the insecticide imidacloprid, which is widely used in agriculture. Results showed that all evaluated enzymes recorded changes in their activity as the imidacloprid concentration increases ( $>0.66$  mg/kg).

The biochemical and genetic toxicity of dinotefuran on *Eisenia fetida* were evaluated at 0, 0.1, 0.5, 1.0, and 2.0 mg/kg in a study conducted by [81]. Dinotefuran induced excessive reactive oxygen species (ROS) generation at 1.0 and 2.0 mg/kg, and it resulted in significant changes in the activity of antioxidant enzymes and on the functional gene expression. Moreover, lipids, proteins, and nucleic acids were oxidized and damaged by the excess of ROS induced by dinotefuran—this process results in serious destruction of cell structure and function.

Research such as those conducted by [79, 80] are promising, but further research is needed in order to explain certain mechanisms, given the complexity of some enzymes, as well as DNA damage and its possible consequences on organisms.

## 5. Conclusions

Natural soil resources must be interpreted and studied by taking into account all their compartments, since they act together in different soil functions.

Contaminated soils have been largely monitored through physical-chemical analyses. However, soil quality assessments and synergistic effects caused by contaminants are not taken into account. Ecotoxicological tests are critical for the evaluation of potential environmental risks posed by these contaminants.

It is important carrying out tests by using organisms that represent terrestrial ecosystems in toxicological evaluations. Toxicity tests are tools that help the management of contaminated areas. They indicate the viability of natural environmental recovery, since organisms in the field degrade and mineralize substances in contaminated environments.

It is recommended to extend the present study to other organisms, such as microcrustaceans, living in different habitat types (sediments and water) in order to cover different trophic levels and to assess whether there is, or not, toxicity transfer between food chain levels.

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