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Chapter 3

Overview of the Standard Methods for Soil Ecotoxicology Testing

Paulo Roger Lopes Alves and Elke Jurandy Bran Nogueira Cardoso

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Abstract

This chapter briefly describes the importance of the services provided by soil invertebrates in terrestrial ecosystems and highlights the role of soil fauna in the risk assessments of potentially polluting substances for the terrestrial environment, considering the sensitivity of these organisms, when compared to other indicators of soil quality (e.g., chemical and physical). The main invertebrate groups used in laboratorial ecotoxicological assays are presented and, based on its physiological characteristics and habit requirements, the advantages and disadvantages of using certain taxonomic groups in laboratory assessments are also discussed. The most frequently used methods to perform this type of toxicity tests are summarized, highlighting the fundamental steps of the assays with the species *Eisenia fetida*/*Eisenia andrei*, *Folsomia candida*, *Enchytraeus albidus*/*Enchytraeus crypticus*, and *Hypoaspis aculeifer*, as well as the possible adjustments that are being carried out in tropical countries. Finally, the future prospects, related to the challenge of increasing the realism of laboratory ecotoxicological analyses, are discussed to show the main needs of this study at global and regional perspectives.

Keywords: Soil invertebrates, Bioassays, Ecotoxicity, Risk assessment, Standard procedures

1. Introduction

1.1. The role of soil invertebrates in soil-risk assessments

Soil represents one of the most complex and diverse ecosystems on earth. In addition to providing the main environmental support for the majority of plants, soil provides the habitat for a vast diversity of animals (vertebrate and invertebrate) and microorganism taxa. Soil is estimated to harbor one-fourth of all of the described biodiversity [1–2]. Although organisms may occur in almost all soil profiles, biological activity is highly concentrated in the most

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superficial layers, mainly within the top 30 cm of soil, where the highest concentrations of organic material are also found [3–4].

The transformations promoted by soil organisms, especially those that benefit human populations, are known as ecosystem services [5]. The concept of ecosystem services is recent, and a large number of services have already been identified as priorities for terrestrial ecosystems because they significantly influence the daily life of human populations and are fundamental for the maintenance of ecosystems and agriculture.

Soil invertebrates are directly or indirectly responsible for various biological and biochemical processes underlying terrestrial ecosystem services, namely, at the level of microbiota regulation, nutrient cycling, soil structuring, and water-quality regulation through filtration processes. These organisms act directly on the fragmentation and distribution of organic material deposited in the soil (animal and plant debris) and function as catalysts of organic matter (OM) decomposition processes and the biogeochemical cycles of carbon, nitrogen, phosphorus, and sulfur [5]. Moreover, these organisms regulate microbial activity (including the control of pathogens), microorganism distribution along the soil profile, and soil structuring because certain fauna species build tunnels, galleries, and other structures along soil profiles (horizontally and vertically) that are used to transport microorganisms and/or their latent forms (e.g., fungal spores) and provide preferential routes for organic matter incorporation and distribution as well as for root growth.

A classic example of a terrestrial ecosystem service provided by soil invertebrates is their influence on the mineralization of nutrients contained in the soil organic matter (SOM), a process that is fundamental for the maintenance of agriculture and forestry systems. This recycling of chemical elements is responsible for the supply of a large portion of the nutrients required by plants as well as increases in the productivity of these systems [6]. Therefore, it is essential to protect the ecosystem services provided by soil fauna.

In order to rapidly meet the high demand for food and products at lower production costs, methods that are more aggressive to the environment are being used, such as the intensive use of pesticides (to control pests and diseases), discharge of agro-industrial waste directly into the soil (without treatment), and other types of exploitation of the edaphic system [7]. These interventions may present a high risk for terrestrial ecosystems because they constitute entryways of several potentially toxic contaminants that may compromise invertebrate performance and their services, thus also affecting soil microorganisms and the functionality of soil ecosystems.

Until recently, the impact of anthropogenic contamination of terrestrial ecosystems has been primarily measured using indicators of soil chemical and physical properties because biological properties have generally been considered more difficult to predict or even measure [8]. The pH, cation-exchange capacity (CEC), organic matter content, and soil nutrient levels (fertility) are the most commonly used chemical parameters for evaluating soil health. However, these parameters are especially relevant when analyzing the soil's capacity to increase crop yield [9]. Similarly, the physical parameters texture, aggregate stability, soil density, and soil porosity, which are simple, fast and lowcostanalyzes [9], can be used as

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indicators of soil health. Nevertheless, they aremainly related to hydrological processes such as erosion, aeration, soil runoff, infiltration rate, and water-holding capacity (WHC) [10].

A large number of traditional studies that have applied environmental impact indicators for terrestrial ecosystems have only utilized the parameters organic matter (as chemical indicator) and soil-aggregate stability (as physical indicator), whereas a smaller number of studies have correlated biological indicators (bioindicators) to soil quality [8]. However, the impact of contaminants on soil may be more easily identified through their effects on biota than from the results of chemical and physical analyses [11–13] because of the higher sensitivity of biological processes and the capacity of organisms to detect and rapidly respond toa particular part of the contaminant concentrations in the soil (e.g., heavy metals), which is the fraction of contaminants available for uptake by living organisms known as the bioavailable fraction [14]. Therefore, assessing the impact of contaminants through their effects on biota may provide an early warning of risks to terrestrial ecosystems [3]. In addition, assessments using living soil organisms, called bioindicators, can be used to determine whether contaminants released into soil affect ecosystem services.

Standardized ecotoxicological laboratory tests using invertebrates are one of the first steps in risk assessments of soil contaminants, and they can be considered one of the main assessment tools because they are frequently sufficient for determining the ecological risk level of substances and the safe exposure limits for humans and soil biota [13, 15]. This type of test provides quantitative and/or qualitative information on the toxic effects of contaminants on soil invertebrate fauna, including information required by several global regulatory authorities prior to the sale of pesticides [16] or to allow the application of residues to agricultural soils [17].

In the European Union (EU), the sale of phytopharmaceuticals (including pesticides) is regulated by specific guidelines that require standardized ecotoxicological tests to assess the impact of these substances on nontarget soil organisms [16, 18]. Regarding the application of agro-industrial residues to soil, the EU requires that assessments be performed according to Directive 2008/98/EC [17], which includes an ecotoxicological risk criterion (H14 – "ecotoxic"). This criterion is used to identify wastes that constitute or may constitute immediate or delayed risks for one or more sectors of the environment (including soil invertebrates) and may be the determining factor used to decide the hazard level of more than 80% of wastes [19].

Two different approaches can be distinguished in laboratory ecotoxicological tests [20]. The first approach involves analyses that are predictive/prognostic and aim to determine the possible toxic effects of the substances tested on invertebrates in case the substances are released into the soil. This approach is mainly used to test new substances (e.g., new pesticides and pharmaceutical drugs) for which the safe exposure levels in terrestrial environments are unclear; thus, this approach can be used to regulate their use or prevent their introduction to the market. The second approach involves analyses that are diagnostic and aim to determine the actual ecological risk or current damage using samples of contaminated natural soils. In this case, the assessments are used to define the priorities for remediating contaminated areas and/or actions for reducing ecological risks.

These two approaches include various tests that can be classified according to exposure time (acute or chronic toxicity), observed effect (mortality, reduced growth or reproduction, bioaccumulation, or behavioral changes) or effective response (lethal or sublethal) [21]. In these tests, representative species of the soil fauna are exposed to increasing contaminant concentrations and the contaminant effects are measured in one (single species) or several species (multispecies) to test dose–response relations [22].

There are various methods for evaluating toxicity in invertebrates, including topical application, force-feeding, and immersion tests [23]. However, the main laboratory assays standardized by the norms of the Organization for Economic Cooperation and Development (OECD) and International Organization for Standardization (ISO) consist of exposing standard species to samples of contaminated soil. These protocols describe methods used to determine acute and chronic toxicity and the effects on the behavior of earthworms, collembolans, enchytraeids, mites, mollusks, and few other insects [20].

The objective of acute toxicity tests is mainly to assess whether a substance causes organism death. These tests are useful for short-term identifications of highly toxic contaminants; however, they do not consider different stages of the test organism life cycle (growth, reproduction, and birth of juveniles) or determine whether particular life cycle stages present increased sensitivity to toxic substances [24]. These tests are also used as preliminary evaluations ("range-finding tests") to determine the concentration ranges to be used in definitive acute toxicity tests and/or the sublethal concentrations for chronic toxicity assays [13].

Chronic toxicity tests are medium-term tests that measure the sublethal effects of potentially toxic substances on organisms, such as changes in reproduction and growth, and are more adequate for assessing effects at the population level [20, 25]. The primary standard methods for laboratory chronic toxicity tests have been established in ISO [26–27] and OECD [28–31] guidelines. The objective of these standardized tests is similar for different groups of invertebrates, although they do present differences, especially in test duration, as a result of the different reproductive characteristics of different species. In these tests, adults are exposed to a range of sublethal concentrations of the test substance, with the concentrations defined according to preliminary tests (range finding) or results from the literature.

Behavioral tests with soil invertebrates are also becoming common because they provide a preliminary evaluation of responses to soil pollution over a shorter period relative to that of toxicity tests [13]. In addition to providing ecologically relevant results because of the sensitivity of species in detecting polluting substances in soils, these tests can be performed more quickly (2 days on average) and at a reduced cost [13, 32]. Avoidance tests, for example, can be used as triage tools to assess the habitat suitability of soils because they are based on the ability of animals (e.g., earthworms) to avoid potentially toxic substances upon exposure to contaminated soils [33] because of the presence of chemoreceptors that are highly sensitive to chemicals in the environment [34].

Although behavioral assays offer alternative endpoints for assessing the impact of contaminants on soil invertebrates, it is recommended to use such evaluations along with acute and/or chronic toxicity tests [35] because in certain cases, the substances can cause 100%

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mortality without the observance of an avoidance effect [32]. Such cases may be related to the test substance's narcotic properties, an absence of irritating effects, or physiological adaptations associated with the species' mode of life [36]. Integrated laboratory analyses should therefore be performed when a higher precision is required when assessing the interactions between contaminants, animal species, and soil properties because such analyses decrease the amount of uncertainty when determining ecological risks.

Although the standardized guidelines for ecotoxicological tests for terrestrial environments are relatively new, compared with the guidelines for aquatic environments, the number of tests based on these guidelines has increased considerably, and such tests have been used to investigate the ecological risk assessment of fungicides, herbicides, insecticides, heavy metals, nanomaterials, agro-industrial residues, and other substances in soil [13, 20].

2. Standard species for soil ecotoxicology

Ideally, the toxic effects of all chemicals introduced to terrestrial ecosystems, such as agro-industrial and urban wastes, should be tested on all species inhabiting the ecosystem before commercialization (xenobiotics) or direct application to the soil [13]. Because these measures cannot be achieved in the laboratory, edaphic invertebrate species that have known sensitivities to anthropogenic changes and provide the main representative ecosystem services of the fauna have been chosen as indicators of the ecotoxicological risk to terrestrial ecosystems. The review presented in Ref. [20] provides a list of the main invertebrates used in terrestrial ecotoxicological assays. This list includes earthworms (*Eisenia andrei* and *Eisenia fetida*), enchytraeids (*Enchytraeus albidus* and *Enchytraeus crypticus*), mollusks (*Helix aspersa*), mites (*Hypoaspis aculeifer*, *Platynothrus peltifer*, and *Oppia nitens*), isopods (*Porcellio scabere* and *Porcellionides pruinosis*), collembolans (*Folsomia candida* and *Folsomia fimetaria*), insects of the family Carabidae (*Pterostichus oblongpunctatus* and *Poecilus cupreus*), *Oxythyrea funesta*, and other organisms used in the methods standardized by international guidelines for ecological risk assessments of soil contaminants.

The methods described by the ISO and OECD international guidelines for ecotoxicity tests with terrestrial invertebrates are designed to standardize the tests so that similar results can be obtained in different laboratories regardless of the region. Such standardization facilitates comparisons and increases the reliability of the established toxicity levels. To develop standardized tests, standard species must be selected based on ecological relevance, ease of maintenance in the laboratory, and short-generation time [37–38]. In addition, the selected species should have well-known biological parameters so that a large number of healthy, homogeneous (same size and biomass), and age-synchronized individuals can be obtained. The number of species that meet all of these requirements is small.

In general, toxicity assays with standard species are performed individually; therefore, each species is tested separately to exclude the effects of interactions among species present in soil. However, the use of several species, even when tested separately, increases the ecological relevance of laboratory analyses because different organisms respond differently to pollutants,

and the potential risk to the ecosystem varies [20]. The performance of a balanced battery of tests using organisms of different functional and taxonomic groups and different routes of exposure is therefore necessary to improve the reliability of ecological risk assessments determined via laboratory tests [39].

The main standard invertebrates used in batteries of soil ecotoxicological tests are earthworms [29, 40–42], collembolans [26, 31], mites [39], and enchytraeids [22, 28, 43]. Because of different morphological (e.g., epidermis structure) and physiological (e.g., water and oxygen uptake pathways) characteristics as well as feeding and behavioral habits (e.g., movement within the soil or at the soil surface and digging habits), these taxonomic groups encompass different pollutant uptake routes and encounter pollutants through the exposure to water and air present in the soil pores and ingestion of food and soil particles [44].

Earthworms are important components of the soil biota because they aid in the formation and maintenance of soil structure and fertility. Although they are not numerically dominant, their large size makes them one of the main contributors to invertebrate biomass in soil. These organisms are important indicators of soil life and quality because their populations are affected by common agricultural practices, especially by the use of pesticides and fertilizers and application of waste [45]. Because they are soft-bodied organisms, earthworms absorb water mainly through their skin; therefore, they can accumulate chemicals during water absorption [44]. Another important route of contaminant absorption is through ingestion because these organisms ingest large amounts of soil with adsorbed substances along with their food (soil organic material, which may also be contaminated).

Although several earthworm species have been used in terrestrial ecotoxicological tests [22], only *E. fetida* and *E. andrei* were included in the ISO and OECD guidelines. These species are preferred because they have worldwide distribution, are naturally tolerant to various organic substrates, are easily handled in single species or mixed cultures [45], and may be easily acquired commercially (adults, juveniles, or cocoons) or obtained from other soil ecotoxicology laboratories.

E. fetida and *E. andrei* were initially differentiated into two forms, var. tipica and var. unicolor (lightly stripped and uniformly pigmented, respectively), by Bouché (1972), who considered them as subspecies (*E. fetida fetida* and *E. fetida andrei*). These organisms were thought to belong to the same species for a long time because of their similar appearances, ecological demands, and frequent associations. However, recent investigations have determined that their crossing does not result in viable descendants, and biochemical methods were used to confirm that they constitute different species [46]. This differentiation is important for their use in ecotoxicological tests because the effects of contaminants on the two species may be different.

Neither of the two species is typical of most agricultural soils, and they only occur in soils rich in organic matter. Under ideal conditions, their life cycle until maturity is relatively short (varying between 45 and 51 days) compared with that of other species and extends from the recently deposited cocoon until the adult stage, when the worms are sexually mature (with the presence of clitellum) and ready to produce the next generation. The time for juveniles (recently hatched) to reach sexual maturity varies between 21 and 30 days. Both species are

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prolific, and between two and five juveniles are generated from each viable cocoon. Depending on the rearing temperature and substrate, their maximum life span ranges from four and a half to five years [45].

Enchytraeids (family Enchytraeidae) belong to the same class as earthworms (Oligochaeta) and can live in both water and soil. However, they are mostly found in soil, where they perform important ecosystem services, such as increasing the rate of organic matter decomposition, maintaining the soil structure (creation of biopores), and dispersing microbes on a local scale [47–48]. These services are especially important in acidic, sandy, and nutrient-poor soils, where enchytraeids are the dominant soil fauna group (up to 75% of the biomass) [48–49]. In these environments, the role of enchytraeids in organic matter decomposition may not be performed by other fauna groups [50].

The family Enchytraeidae has over 600 described species [48]. Known as white worms or potworms because of their pale color and small size (many are only a few millimeters long, although some may reach up to 5 cm), most enchytraeids are hermaphroditic (capable of self-fertilization), although certain species are parthenogenetic or reproduce through fragmentation [51]. Enchytraeids have a limited capacity for movement inside the soil; therefore, they live in the most superficial soil layers (0–10 cm) where the organic material and biological activity are concentrated. These organisms are found from arctic to tropical regions, and they are more abundant in forest soils (or soils rich in organic material) and less abundant in pastures and agricultural fields [52]. Their main food source is fungal mycelium; however, they also feed on organic matter that has been predigested by fungi as well as on other microorganisms [48].

These oligochaetes are sensitive to potentially toxic substances abundant in many soils where earthworms are not present or are not well represented. In addition, these animals are easy to handle and rear and have a significantly shorter life cycle than other worms, which is convenient for standardized toxicity assays [28, 43, 53]. These organisms live in close contact with the soil pore water and are exposed to soil contaminants through dermal, intestinal (through feeding), and respiratory routes [44]. Although their use in laboratory ecotoxicological tests was reported for the first time approximately 40 years ago, enchytraeids were selected for use in standardized ecotoxicological laboratory tests only 10 years ago as reported in the guidelines ISO 16387 [43] and OECD 220 [28].

Enchytraeus is the only enchytraeid genus with species selected for ecotoxicological tests standardized by ISO and OECD guidelines (e.g., *E. albidus* and *E. crypticus*) because this genus is considered typical of environmental stress indicator organisms and can be easily reared in the laboratory. *E. albidus* is the best known species for soil ecotoxicology [54] because it can be reared in many different substrates (it is widely distributed in terrestrial ecosystems) with different types of food, and it has a proven sensitivity to soil contaminants [55]. When these animals reach maturity (approximately 21 days at 18°C), the size of *E. albidus* adults can vary between 15 and 40 mm. Variations are observed in the total developmental cycle (33–74 days), embryonic development period (12–18 days), eggs per cocoon (7–10), and viable cocoon percent (40–50%) according to environmental conditions, especially in response to changes in culture temperature [43, 54]. The ideal reproduction temperature for the species is 15°C,

although they can reproduce at temperatures between 12 and 22°C. Temperatures above 25°C should be avoided because they can suppress reproduction [54].

Although *E. crypticus* has lower ecological relevance because its prevalence in the field is unknown [56], the species is adequate for ecotoxicological laboratory tests, and its use in current standardized tests appears to be increasing [20]. Compared with *E. albidus*, *E. crypticus* has the advantage of being able to grow in agar medium, and it also has a higher reproduction rate, has a shorter generation time, tolerates a wider range of soil properties, and presents other characteristics considered methodologically advantageous [53, 57]. The adults of this species vary in sizes between 3 and 12 mm, and they have a generation time of approximately 18 days (at 21°C) in agar medium. The mean number of eggs per cocoon can vary between 1 and 35 with a mean of 4.6 eggs produced per day [58]. Studies have indicated that the number of juveniles may also vary according to the type of soil used, with apparently higher numbers in standard LUFA 2.2 natural soil [59] than in artificial OECD soil [60].

The order Collembola is one of the most diverse and abundant terrestrial arthropod orders, with 21 families and 20,000 described species [61]. In general, collembolans are small, varying from a few to approximately 10 mm in length [62]. The body can exhibit colorful pigmentation, although the inhabitants of deeper soil layers are typically not pigmented [63]. Most species feed on fungal hyphae and decomposing plant material; thus, they have a significant effect on microbial ecology and soil fertility and can control certain plant diseases caused by fungi [62, 64].

Collembolans are vulnerable to the presence of potentially toxic contaminants in soil [3] because they are exposed through water ingestion or absorption from wet/moist surfaces, food (living or dead) consumption, and soil pore air inhalation [44]. The responses obtained in tests using these arthropods may indicate environmental stress levels and the ecological risk of substances; therefore, these organisms supply information that can serve as a basis for legislation [65]. Collembolans have been used to estimate the effects of pesticides and other environmental pollutants on nontarget soil arthropods for approximately four decades, with *F. candida* – being typically used in standardized ecotoxicological tests [26, 31, 66]. *F. candida* has great importance for terrestrial ecosystem services because of its high sensitivity, short generation time, high reproduction rate, and easy culturing in the laboratory using a diet of granulated dry yeast [67].

F. candida (Willem 1902) is an arthropod of the family Isotomidae, and it is distributed in soils worldwide, although it is not common in most agricultural soils. This animal has a high occurrence rate in sites rich in organic matter [68]. This species has no pigmentation or eyes [38], and it reproduces exclusively through parthenogenetic females, which are approximately 2 mm long and sexually mature at 21–24 days of age (at 20°C). The optimal temperature for egg incubation and production is 21°C, and under these conditions, females lay 30–50 eggs, which take 7–10 days to hatch. This species has strong feeding preferences for certain species of fungi, and they are classified as microsaprophagous [62] and can be fed dry yeast in the laboratory [26, 31].

Although used in a fewer number of studies, *F. fimetaria* has been used as complementary or alternative species to *F. candida*, and the choice to use *F. fimetaria* is related to its higher

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ecological relevance because it is present in many natural and agricultural habitats where *F. candida* is not found. In addition, *F. fimetaria* meets all of the necessary requirements of a standard species for ecotoxicological laboratory tests [69]. In 2009, OECD guideline no. 232 [31] established *F. fimetaria* as a standard species for standardized laboratory assays. In addition to *F. fimetaria*, other species can be used to increase the ecological relevance of tests, although they may not be included in guidelines [21, 68, 70–71].

Mites are arthropods belonging to class Arachnida and subclass Acari, and they have a small size and unsegmented bodies [61]. In total, mites are ordered in 1,200 families and approximately 500,000 species [72], of which many are the most abundant mesofauna inhabitants in many types of soil and litter [73]. Suborder Gamasida (order Mesostigmata) includes the main species of predatory mites inhabiting soil pores [74]. The community structure and abundance of predatory mites are strongly dependent on the nature and availability of their prey [22]. Most mites feed on enchytraeids, nematodes, and microarthropods, although certain groups are considered fungivorous, bacteriophagous, facultative phytophagous, or have unknown feeding habits [73].

The ecosystem services provided by soil predatory mites include the biological control of pests and other species with abundant populations; thus, they significantly contribute to the flow of energy and matter in terrestrial ecosystems as well as to the maintenance of food chains [22]. In addition, several genera have been isolated for over 30 years from soil and tested as quality bioindicators [72]. Currently, reports are available for different toxicity tests using these arthropods; however, only the reproduction test using *H. aculeifer* has been standardized by guidelines for the evaluation of soil quality [20, 22, 30]. *H. aculeifer* has been considered the most adequate mite species for ecotoxicological assays because it has an acceptable generation time (approximately 1 month at 20°C), is a generalist predator, and can be easily handled in the laboratory [20, 72]. Because it represents a different trophic level from the other invertebrates used in standardized tests and is exposed to contaminants through different routes [44], *H. aculeifer* has been included on the EU community's list of nontarget organisms considered in the assessment of environmental risk of pesticides in soil [16, 72, 75].

Specimens of *H. aculeifer* are brown and have a light-brown dorsal shield. Although the size of both sexes is rather small, females are larger (0.8–0.9 mm) than males (0.55–0.65 mm) [22, 76]. Under temperatures between 20 and 23°C, these organisms become adults in approximately 16 (females) and 18 (males) days after going through larval, protonymph, and deutonymph developmental stages. However, their development time can be strongly affected by the temperature [76]. Usually, reproduction is sexual, although arrhenotokous parthenogenesis may occur in the absence of males, with this process only generating males [77]. However, females generally occur at a higher frequency because the sex ratio can also be controlled through selective cannibalism [78]. Each female lays approximately 100 eggs during its reproductive life [79]. These eggs are white, elliptical, and laid at the soil or culture substrate surface. Although it is a polyphagous predator, it is usually fed with the mites *Tyrophagus putrescentiae* or *Rhizoglyphus* sp. in ecotoxicological tests [30]. In the case of food scarcity, these organisms can survive cannibalistically [78].

In Ref. [22], the main invertebrates used in standardized terrestrial ecotoxicological tests were compared and it was concluded that the standard species of earthworms (*E. andrei* and *E. fetida*), enchytraeids (*E. albidus* and *E. crypticus*), collembolans (*F. candida* and *F. fimetaria*), and predatory mites (*H. aculeifer*) are adequate for the performance of tests on several soils from temperate and tropical regions. However, earthworms present limitations in acidic or basic soils. Enchytraeids are more tolerant to changes in soil pH and organic-matter concentrations, although they grow better in sandier soils. Mites and collembolans appear to be adequate for tests in most soil types and are considered less sensitive than oligochaetes (earthworms and enchytraeids) to soil properties. In tests that use soils with extreme characteristics (acidic or sandy soils), more than one species as well as alternative species should be used [22]. In addition, *H. aculeifer* generally appears to be less sensitive to certain substances compared with the remaining species. However, because it is the only predatory standard species, its inclusion in routine assessments of ecotoxicity is supported [80].

3. Summary of the standard procedures for bioassays with soil invertebrates

Performing ecotoxicological laboratory tests requires a series of steps, including planning and material preparation, animal rearing and maintenance in the laboratory, contamination of artificial/natural soils (or preparation of previously contaminated soil samples), experimental procedures for the initial test conditions as well as the maintenance and evaluation of toxicity tests using organisms, and data analysis and interpretation. The steps of the standardized tests using the species *E. andrei*/*E. fetida*, *E. albidus*/*E. crypticus*, *F. candida*, and *H. aculeifer* described in the guidelines ISO no. 11268-2 [27], no. 16387 [43], no. 11267 [26] and OECD no. 226 [30], respectively, will be summarized in this section to describe the main methods established by the guidelines and the adaptations that are used in regions with tropical climates.

Most standardized tests using soil invertebrates were developed to quantify the impact of chemical exposure on organisms in artificial soils [42]. However, although studies using artificial soils supply information that can be internationally compared, natural soils may provide information on local problems. Although the practice is still not described by the guidelines, the use of natural soils in standardized ecotoxicological tests has been increasing.

OECD soil is a standard artificial substrate recommended by ISO/OECD guidelines for most terrestrial ecotoxicology studies [42]. This substrate consists of a mixture of 70% industrial sand (with more than 50% particles between 0.05 and 0.2 mm), 20% kaolinite clay, and 10% peat (ground and dry). However, for assays in tropical regions, studies have used a modified version of this substrate [81–83] known as tropical artificial soil (TAS). TAS uses powdered coconut husks as replacement for peat because of its higher availability in tropical regions. In both cases, after the materials are mixed, the pH (1 M KCL 1:5 weight:volume ratio) of the artificial soil should be adjusted to 6.0 ± 0.5 through the addition of $CaCO_3$. In addition, the soil water-holding capacity should be determined for moisture adjustments [26].

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To use natural soils in standardized tests, the soils should offer minimum conditions for the survival and reproduction of the test species without causing morphological or behavioral changes in the absence of the contaminant. To test chemicals for regulation purposes (e.g., ecological risk of pesticides), natural soils are artificially contaminated, and the results should be comparable between laboratories. Therefore, the soils should have similar characteristics, as is the case for LUFA soils [84–85], EURO soils [86], SIM soils [87], and other natural soils selected as standard soils for specific regions, for example, Polish [88] and Mediterranean soils [89]. In the case of natural soils from contaminated areas, the use of a control soil with the same characteristics (texture, pH, organic matter concentration, and C:N ratio) but without contaminants is recommended [87].

Natural soils should be dried, sieved (5 or 2 mm, preferentially), and chemically and physically characterized before use, and at least the texture, pH, WHC, and moisture content should be determined. In addition, the organic matter concentration (or organic C), CEC, C:N ratio, and metal (or other element) concentration may also be measured [87]. Regardless of the type of soil (artificial or natural), defauning is recommended (by soil freezing at −20°C followed by thawing to room temperature) to eliminate the original soil fauna organisms [90].

According to the guidelines, incorporating test substances directly into soil (artificial contamination) varies with the water solubility of the contaminant, and there are three main methods: (a) for water-soluble substances; (b) for water-insoluble but organic solvent-soluble substances; and (c) for water-insoluble and organic solvent-insoluble substances. In all cases, the concentration gradients of the test substances should be prepared immediately before the beginning of the assay in the volume necessary to maintain the soil moisture between 40 and 60% of its WHC. In the case of water-insoluble substances, after applying the solutions in increasing concentrations, the soil moisture should be adjusted through the addition of pure water. It is recommended that the concentrations be prepared in a geometric series separated by a factor of 1.8 or lower. If effects are not observed for the tested substance (e.g., active ingredient of a pesticide) at the highest concentration (1,000 mg kg^{-1}) in the preliminary tests (acute toxicity assays), then a limit test should be performed to evaluate the toxicity using only the control treatment at a concentration of 1,000 mg kg^{-1}.

The environmental conditions of the standardized ecotoxicological tests and laboratory cultures should be controlled. Controlled temperatures and light conditions in the test/culture chamber/room are fundamental for obtaining homogeneous cultures (with the same age and size) with development cycles that occur within the time predicted for the tests. The main recommended protocols are a mean temperature of 20 ± 2°C and constant light intensity between 400 and 800 lux on the culture containers [26, 31]. In addition, it is recommended that cultures be kept under controlled light/dark cycles, preferably 12 hours light/12 hours dark or 16 hours light/8 hours dark. However, the environmental test and culture conditions have been adapted for regions with predominantly tropical climates to better reflect the influence of local climate conditions. To simulate tropical conditions, temperatures varying between 23 and 27°C have been used [83, 91–93].

The earthworms *E. andrei* and *E. fetida* should be reared in substrates composed of a mix of horse or cow manure (defauned following the same process described for soils) and peat (1:1,

dry weight) [27], and the culture medium should be moistened weekly with pure water. Similar to artificial soils, peat is usually replaced with powdered coconut husks in tropical climate regions [13, 18, 81]. Earthworms should be fed weekly with a mixture of oat flakes and water, and the rearing substrate should be periodically replaced.

E. crypticus can be cultured in natural soil, although recent studies have opted for culturing in Petri dishes with agar medium [83, 94]. This culture medium is composed of a mixture (1:1, v:v) of a salt solution (calcium chloride, magnesium sulfate, potassium chloride, and sodium bicarbonate) and a bacto-agar solution (e.g., Oxoid - Agar No. 1). Approximately 50 mg of ground oat flakes should be supplied as food once a week, and the organisms should be transferred to new substrate every 2 months.

Laboratory cultures of collembolans and mites should be performed using a substrate composed of a mixture of activated charcoal, plaster of Paris (calcium sulfate), and deionized water, with a recommended 1:8 charcoal:plaster of Paris ratio for *F. candida* and 1:9 ratio for *H. aculeifer* (w:w) [26, 30]. The volume of deionized water should be 60–100 mL for each 100 g of mixture, although the water content varies with the type of plaster of Paris. The bottom of plastic containers should be filled with the mixture to a height of approximately 1 cm. Collembolans should be fed dry yeast once a week, and cultures of *T. putrescentiae* or *Calogly-phus* sp. (cheese mites) should be simultaneously maintained with *H. aculeifer* cultures to serve as food (prey) for the predator. Small quantities of cheese mites should be supplied to *H. aculeifer* twice a week and the cheese mites should be fed once a week with powdered brewer's yeast [30].

Avoidance assays with *E. andrei*/*E. fetida* or *F. candida*, which are described by the guidelines ISO:17512-1 and ISO:17512-2 [41, 95], are performed using rectangular (earthworms) or round (collembolans) plastic boxes that are divided into two equal compartments by a plastic divider vertically introduced and filled with the test soil (amounts depend on the size of the container). Contaminated soil is added to one of the compartments, and the same amount of the respective control soil is added to the other compartment. Ten *E. andrei* or *E. fetida* adults (with developed clitellum) or 20 *F. candida* individuals at 10–12 days of age (originating from synchronized cultures) are then placed on the separation line between the two compartments, and the plastic divider is removed. The containers are then closed with perforated lids to allow air circulation. The animals are not fed during the test. After 48 hours, the number of individuals present in each compartment is recorded according to the specific method for each species [18, 96]. A double control test is also performed with control soil in both compartments to determine whether the organisms are randomly distributed between the two compartments in the absence of contaminants.

Chronic toxicity tests with *E. andrei*/*E. fetida* should be performed according to ISO:11268-2 [27]. Round plastic containers are filled with approximately 500 g soil (dry weight) treated with solutions with increasing contaminant concentrations (or dilutions of the naturally contaminated soils) or the respective control soils. Ten adult earthworms (with visible clitellum) with individual weights between 300 and 600 mg that had been previously incubated in control soil for at least 24 hours are selected for each experimental unit. The containers are closed with perforated lids, and the earthworms are fed horse manure (≈5 g per replicate) at the beginning

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of the assay and then once a week until the end of the assay. The assay lasts for a total of 56 days. Adult earthworms are removed after 28 days, washed with water, and weighed. The final percentage of body biomass (after 28 days) relative to the initial weight can be calculated to assess the effects of contaminants on organismal growth. After 56 days from the beginning of the assay, the number of juveniles is counted to verify the treatment effect on species reproduction (for additional details, see Ref. [27], and/or Ref. [18]).

Reproduction tests with *E. crypticus* are described in ISO:16387 [43]. Ten similar-sized adults (with visible clitellum) are placed in cylindrical containers containing 20 g soil (dry weight) that had been treated with the test substance or the respective control soil. Finely ground oat flakes can be supplied as food (≈2 mg per replicate), and the containers should be hermetically closed [83]. The containers are opened weekly to allow for gas exchange, and food and soil moisture are replenished as needed. Twenty-eight days following the beginning of the assay, the total number of enchytraeids are counted using a stereoscopic microscope following fixation in 80% ethanol, staining with rose bengal (1% in ethanol), and wet sieving of the organisms [94]. For *E. albidus*, the experimental procedures are somewhat different, especially with regard to the assay duration.

Tests evaluating the impact of contaminants on the reproduction of the collembolan *F. candida* are described in ISO:11267 [26]. Thirty grams of contaminated or control soil (fresh weight) are added to cylindrical containers (approximately 100 mL). Ten adult collembolans aged between 10 and 12 days (originating from synchronized cultures) are then placed into each experimental unit, and the containers are then hermetically closed. Food (dry yeast) is supplied at the beginning of the assay and on the 14th day, and the containers are opened weekly to allow for gas exchange. On the 28th day after the beginning of the assay, the soil of each replicate is submersed in water to force the survivors to float to the surface, and the juveniles are counted following the addition of several drops of black ink (for increased contrast) [93].

Reproduction tests with *H. aculeifer* are described in guideline OECD no. 226 [30]. The containers are filled with 20 g (fresh weight) of soil treated with the test substance or control soil. Ten females with ages between 28 and 35 days and originating from the synchronized cultures are then placed in each container. The animals receive small amounts of food (cheese mites) at the beginning of the assay and then twice a week until the end of the assay. The containers are then hermetically closed and opened weekly for airing and soil moisture adjustments. Fourteen days after the beginning of the assay, the mites are removed from the soil using a MacFadyen extractor with a gradient of increasing temperatures for 48 hours (12 hours at 25°C, 12 hours at 35°C, and 24 hours at 45°C). Adults and newly emerged juveniles in each replicate should be fixed in 70% ethanol and counted using a stereoscopic microscope.

The following parameters are used to evaluate the critical values: NOEC (no observed effect concentration) and LOEC (lowest observed effect concentration), LC_{10} and LC_{50} (lethal concentration to 10 and 50% of the population, respectively), EC_{10} and EC_{50} (concentration at which 10 and 50% of the contaminant's maximal effect is observed on the growth or number of juveniles, respectively), and AC_{50} (concentration causing the avoidance of 50% of the organisms from the contaminated soils). These toxicity parameters are designed to detect the

ecological risk of the chemicals in soil and used to derive protection limits for terrestrial ecosystems.

The significance of avoidance responses (LOEC and NOEC) is tested with Fisher's exact test using a two-tailed test for the double control conditions and a one-tailed test for the contaminated soil combination conditions [97]. The AC_{50} values can be obtained using regression analyses [98]. The significance of the effects (LOEC and NOEC) on the body biomass of the earthworms and number of earthworms, enchytraeids, mites, and collembolan juveniles following exposure to the contaminated soils should be tested using a one-way analysis of variance (ANOVA). When differences are detected ($p \leq 0.05$), the treatment means should be compared with the results from the respective controls using a post hoc test such as Dunnett's test. The EC_{10} and EC_{50} values should be estimated by nonlinear regression using pre-defined exponential, logistic, Gompertz, or hormesis models [71]. The normality and homogeneity of variance should be tested prior to the ANOVAs.

4. Future prospects in soil ecotoxicology

The future prospects for soil ecotoxicology refer to the challenge of increasing the realism of the analyses in the terrestrial environment and to reduce the uncertainties about the real degree of ecological risk obtained by laboratory tests. To improve the ecological relevance of laboratory assays, it needs a transition from research based on artificial soil to the use of natural soils, which consider the real relationship between contaminants and test organisms in the exposure scenarios. Moreover, it is necessary to increase the list of standard organisms for the tests, considering the practicality in assays, and especially the inclusion of species that represent the geographical and ecological conditions of the test site [99]. As an example, Ref. [20] suggests the standardization of the sublethal toxicity tests with isopods, since they represent an ecologically relevant group of soil fauna, and the effects on these arthropods can be determined at biochemical, genomic, individual (growth, behavior), and ecological (feeding activity) levels.

It is still necessary to move forward in the assessments of long-term sublethal effects; besides, there is a need for better understanding of exposure, absorption, and metabolism of substances in individuals, and the identification of the responses at different levels of biological organization (e.g., communities) [20]. Based on this assumption, one of the apparent possibilities to evaluate the long-term impact in standard laboratory tests would be the use of multigenerational assays, where toxic effects such as delayed reproductive failures, transmission of the bioaccumulation to offspring, or accumulation of DNA damage could be identified [100]. For a better understanding of the relationships between pollutants and species, more studies using chemical, biochemical, and molecular prospects (ecotoxicogenomics) are needed, particularly assessments of bioavailability, bioaccumulation, and molecular biomarkers [20, 101]. The assessment of impacts at higher levels of biological organization can be accomplished through multispecies assays, which consider the relationships between species, contaminants, and soil properties. In addition, the semi-field and field tests may offer a better understanding of the contaminant's impacts on soil communities, although they are more complex, especially when there is the involvement of comparisons between different ecosystems [13, 20].

Finally, it is necessary that future adjustments be performed in the standard assays available, in order to enable them to address the new and emerging needs of the current ecotoxicology, such as the case of evaluation of the toxicity of nanoparticles and mixtures of contaminants, among others [20]. These adjustments also extend to the studies performed in tropical regions, where there is a need for a revision of the methods, especially in terms of soils, species, and climatic conditions, in order to increase the ecological relevance of the analyses at local level.

Author details

Paulo Roger Lopes Alves[1*] and Elke Jurandy Bran Nogueira Cardoso[2]

*Address all correspondence to: paulo.alves@uffs.edu.br

1 Federal University of South Border, Chapecó, Brazil

2 Luiz de Queiroz College of Agriculture -University of São Paulo, Piracicaba, Brazil

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