Section I: Terrestrial Invertebrates as Experimental Models

CHAPTER 1

The Use of Non-standardized Invertebrates in Soil Ecotoxicology

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1.1 Soil Invertebrates

From the approximately 8.7 million species estimated to be living on planet earth, about 7.7 million are represented by individuals of the animal kingdom,[1] and only about 5% of these animals are represented by those who have a backbone, known as vertebrates. All the others, representing the major part of the animal kingdom, are known as invertebrates.[2] In general, invertebrates are multicellular animals that do not have and do not develop a vertebral column derived from the notochord.

All existing invertebrates are distributed through about 35 phyla, but the number of phyla may vary according to the chosen classification. Most of

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them belong to aquatic environments (especially the marine one), although there is a considerable percentage that inhabit terrestrial ecosystems.[2] It is estimated that the animal species living in the soil (including those that spend at least a part of their lives in soil) represent 23% of the total described species of the terrestrial environment (considering all kingdoms). However, more than 22% are represented exclusively by invertebrates,[3] which shows that soils comprise a high diversity of invertebrate species.

Although the diversity of species in the soil can be quite variable, when considering the different types of soil and climate conditions, the majority of the species of soil invertebrates are distributed among the phyla Arthropoda, Mollusca, Porifera, Cnidaria, Platyhelminthes, Nematoda, Annelida and Echinodermata.[2] However, approximately 85% of all of these invertebrates belong to the Arthropoda phylum. Therefore, to make a weighted representation of the invertebrate diversity in soils, this should mainly consist of Insecta, Arachnida, Mollusca, Diplopoda, Annelida and Nematoda,[2] with the highest species richness in those taxonomic groups.[2]

Because it is the easier procedure, for a long time classification of soil animals has been based on body size (length and width).[4] According to several authors,[5,6] the following groups were established as a result of this classification: soil microfauna (less than 0.1 mm), mesofauna (0.1 to 2 mm), macrofauna (2 to 20 mm) and megafauna (larger than 20 mm). The majority of the soil invertebrates can be found in the groups of micro-, meso- and macro-fauna, while megafauna is only represented by insectivorous vertebrates, rodents, as well as some larger sized invertebrates.[2]

This classification includes animals with life cycles ranging from a few days to more than 10 years and it is known that the smaller (micro- and meso-fauna) or the larger organisms (macro- and mega-fauna) are generally related to different specific functions in the soils.[5,7] Therefore, the role of the microfauna is to act as controllers of the microbial soil populations, while the role of the macrofauna is the fragmentation and distribution of the organic material along the soil profile. However, it is important to understand that there is no direct correlation between the size of a soil animal and its trophic position in the food chain.[2]

Soil microfauna is composed of microscopic animals (<0.1 mm), which are the most abundant invertebrates on earth. This classification of small-sized animals encompasses protozoans (Protozoa), nematodes (Nematoda), rotifers (Rotifera) and tardigrades (Tardigrade), among others, which normally inhabit the water film in the soil pores. In general, these organisms have short life cycles and belong to different functional groups, which are classified in accordance with their feeding preferences, such as those that feed essentially on other animals (parasites/predators), on plant roots (phytophagous) or on micro-organisms (*e.g.* fungivorous and bacterivorous). In this sense, during their activities the microfauna stimulate nutrient mineralization as well as the control of microbial populations in the soil.[7,8]

The soil mesofauna comprises invertebrates with body diameter (in width) between 0.1 and 2 mm.[5] In this taxonomic class of animals we find small enchytraeids (Enchytraeidae), pseudo-scorpions (Pseudoscorpionidae), mites (Acari), springtails (Collembola), Diplura, Protura, symphylans (Symphyla), pauropodas (Myriapoda), insects (*e.g.* micro beetles and ants), spiders (*e.g.* small individuals of the family Araneida), and other small arthropods.[7] In general, the predominant eating habit of the mesofauna is detritivorous (feed on litter), fungivorous and/or predators (feed on fungal hyphae and/or on individuals of microfauna, especially nematodes and protozoa). When feeding on litter, these invertebrates expand the contact surface of the organic materials, favoring the microorganism's attack and, consequently, improving the decomposition rates and the nutrient mineralization that favor the plants. Therefore, although the individuals of the mesofauna do not strongly contribute to the overall soil biomass and respiration, they play a key role as regulators of decomposition processes.[9]

Macrofauna encompasses more than 20 taxonomic groups with a body size between 2 and 20 mm,[5] as described below. They are earthworms (Oligochaeta), termites (Isopotera), ants (Formicidae), centipedes (Chilopoda), millipedes (Diplopoda), cockroaches (Blattodea), spiders (Arachnida), earwigs (Dermaptera), crickets (Orthoptera), snails (Gastropoda), scorpions (Scorpiones), stink bugs (Hemiptera), cicadas (Cicadoidea), woodlice (Isopoda), moths (Thysanura), flies (Diptera) and butterflies (Lepidoptera) larvae, and adult beetles (Coleoptera), among others.[2,5,7]

The soil macrofauna eating habits can be quite varied.[7] They can be soil consumers (geophagous), or feed on living parts of plants (phytophagous) including roots (rhizophagus), or on soil organic matter (humivorous), on litter (detritivorous), on wood (xylophagous), on other animals (predators, parasites or necrophagous) or on fungal hyphae (fungivorous). Regardless of their feeding habits, soil macrofauna perform a key role in fragmenting and transporting the organic material (vegetable and animal debris) through the soil profile. In addition, during their transportation activities in the soil profile, most of them (especially termites, carabid beetles, ants, millipedes and earthworms) create biogenic structures (galleries, nests, chambers or fecal pellets), which may modify the physical properties of soils, as well as change the availability of food resources for other organisms. Therefore, they are also called "Ecosystem Engineers".[7,8,10]

Megafauna consist of animals with a body width bigger than 20 mm and are almost entirely vertebrate animals. Therefore, sometimes they are called "Soil Vertebrates".[5] Among the representative species of the megafauna are small mammals and some rodents, amphibians and reptiles, in addition to a few larger invertebrates, normally represented by giant earthworms.[5] Though some larger mammals (*e.g.* hares, rabbits, hedgehogs and foxes) can build their burrows in the soil, they are not considered part of the soil megafauna.[5] With the exception of earthworms, which are geophagous, the megafauna has predominantly a predatory alimentary habit, feeding on smaller invertebrates (macro- and meso-fauna) and on parts of living plants (*e.g.* leaves, stems, roots, seeds). During their activity, the megafauna create great galleries and structures in the soil profile, though in much smaller numbers than the macrofauna.

1.2 The Use of Invertebrates in Soil Ecotoxicology

As already shown in the previous section of this chapter, unlike plants that produce their own energy through photosynthesis, soil invertebrates need to extract the energy needed for survival from other living organisms. This is why they feed on a great diversity of autotrophic, as plants, heterotrophic, as animals, and microorganisms, and occupy several positions in the food web. This fact, added to other varying behavioral habits of invertebrates in the soil, may result in significant differences for the maintenance of terrestrial ecosystems.

The changes promoted by soil organisms, especially those that benefit human beings and the environment, are known as ‘‘Ecosystem Services’’.[11] The importance of the services provided by soil invertebrates in terrestrial ecosystems has been extensively discussed in the literature over the last decade,[3,7,11,12] especially of those who benefit the agricultural ecosystems (agriculture and forestry).

Anthropogenic activities often produce negative impacts on the environment, for example, the impacts of conventional agricultural practices, such as pesticide application, use of chemical fertilizers and waste disposal, among others. They cause stresses on the living organisms of the terrestrial ecosystems and produce negative effects on some of the essential soil ecosystem services.[11] For this reason, researchers in the areas of soil health and soil quality have been warning about the imminent need for developing effective techniques to identify, quantify and prevent the impacts of toxic substances or elements on soil invertebrate species and thus establish limits to protect their associated ecosystem services.

Nevertheless, only in recent years have the evaluations of the ecological risk of contaminants on soil health started to use living organisms as indicators (bio-indicators), as substitutes or complementary to the traditional chemical and physical analyses.[11] For a long time, scientists believed that the biological properties of soils were more difficult to predict or even to measure.[13] However, bio-indicators can be practical tools in this type of assessment because they are highly reactive to the environmental conditions.[14] Changes caused by toxic substances in soil are perceived quickly by analyzing the changes of the living soil community in their presence. Diversity, distribution and vital functions of soil inhabitants as well as the soil’s chemical composition allow us to draw conclusions regarding the quality of contaminated soils. Therefore, nowadays, there are in use numerous protocols of studies using species and communities of soil invertebrates as biological indicators of the impacts of the contaminants in terrestrial ecosystems.

A recent study describes ecotoxicology as a scientific area that studies the effects of chemicals on living organisms in the environment with the final goal of protecting the structure and function of the ecosystems.[15] Thus, methods using soil invertebrates to assess the toxicity of substances disposed on soils are known as Soil Ecotoxicological Assays. This type of testing is based on the principle that the exposure of living organisms to stressful factors, for example those generated by toxic substances, has a particular tendency to change some vital functions (at different levels), allowing us to measure the toxicity of the substances on species, populations and even communities.

Ecotoxicological tests with soil invertebrates can be applicable to many classes of contaminants, may be performed in a short time (for example, in 48 hours), and may be a low-cost option. These tests allow for assessment of a wide range of toxicity types and biochemical, physiological, morphological and behavioral endpoints on organisms.

They have been used to detect the presence, concentration and mode of action of a soil contaminant on the soil organisms and to identify changes and early signs of pollution in the terrestrial environment. Furthermore, they have been used to determine cause–effect and dose–response relations between toxic agents and selected species, to complement the chemical and physical soil analyses in order to indicate the quality of terrestrial ecosystems; and finally, to evaluate the effectiveness of bioremediation management in polluted soils.[16,17]

In general, the aim of soil ecotoxicological assays can be achieved by assessing the toxic effects of chemicals/elements by using single-species tests, under controlled laboratory conditions. Based on the effect concentrations obtained in these tests, it is possible to establish safe limits of exposure for populations and communities living in natural environments. Especially in the case of the prospective risk assessment of chemicals, laboratory tests show a clearer causal relationship between exposure and effects than field assays.[18]

In the case of laboratory tests, the key measuring parameters on invertebrates are related to survival (the number of living/dead individuals), growth (the body biomass or animal body length), reproductive success (the ability to generate viable individuals) and behavioral disorders (as walking ability and avoidance behavior) owing to exposure to contaminants.[11] There are studies based on more complex methodologies on micro- and mesocosm levels, or at field levels, in order to reduce the uncertainty about the toxic risk on invertebrate soil species.[17] In this type of assay, it is possible to evaluate changes in the activity, abundance and diversity of natural soil invertebrate communities from terrestrial ecosystems, and to also assess the direct impact of pollutants on fundamental ecosystem services of human interest. One example is the use of litter-bags to evaluate the consumption of plant material deposited on the soil by the invertebrate fauna in contaminated sites.[19] However, such methods may be expensive, require more labor, are time-consuming and need an integrated analysis of the risk factors.

Although the use of invertebrates to assess the toxicity of substances in soil has been reported since the 1960s,[15,20,21] it was only about 30 years ago that the first standard protocols describing methodologies for laboratory toxicity tests with soil invertebrates were published.[11] According to a literature review of the history of soil ecotoxicology, these international protocols have emerged to support the risk assessment procedures of chemicals in soil as well as to assist in pesticide registration in some countries; consequently they got greater attention of investigators in the related study areas.[15]

Currently, soil ecotoxicology is booming, a fact that can be confirmed when looking at the number of published articles indexed in the database ''ISI Web of Science'' under the terms ''Soil'' + ''Ecotoxicology'' over the few past years. In 2015 this number was about 30 times higher than that of publications in 1992. According to recent literature reviews on this subject, this increase in scientific production is related, among other factors, to a great development in soil ecotoxicology in recent years, highlighted both by the new lines of research in the area and by improvements on traditional laboratory toxicity tests.[11,15,22,23]

Among the new lines of research and of improvements in traditional techniques we include: changes in the constituents of artificial soil[24,25] proposed by the OECD,[26] as well as the replacement of artificial soils (original or modified versions) by natural soils, for example, LUFA soils,[27] and changes in the laboratory climatic conditions (*e.g.* temperature) for species cultures and assays, in order to increase the realism of studies performed under tropical climatic conditions,[24,28,29] and the development of new types of toxicity tests, in order to observe different endpoints (*e.g.* avoidance behavior assays with earthworms and springtails[30,31] are included). Evaluation of the toxicity of mixtures of contaminants and the assessment of the influence of climate changes on the toxic potential of substances against soil invertebrates[32,33] are also included. Finally, with even greater importance for this chapter, research looking for new alternative species of soil invertebrates for the standard laboratory toxicity assays,[34–39] or those with the unique aim of increasing the ecological relevance of non-standardized assays carried out to obtain more accurate responses about specific local ecosystems, play increasingly important roles.

The selection of new test species is particularly pertinent for soil ecotoxicology when taking into account the consistent demand for increasing the representation of the existing taxonomic groups of soil invertebrates in toxicity testing. According to some authors,[12,17] in an ideal situation, the toxicity of all substances deposited on soils should be measured on all animal species from a particular ecosystem, prior to establishing a limit of generic exposure to preserve the invertebrate's biodiversity. However, these authors themselves recognize that this is an impossible task to fulfill through laboratory toxicity tests, if one considers that the diversity of species in soil macro-, meso- and micro-fauna is in the order of magnitude of millions, as described in the first section of this chapter.

Despite the large numbers of taxonomic groups available to be used as bio-indicators for toxic effects of substances in soil,[15,35] only a few species of earthworms, enchytraeids, mites, collembolans, nematodes, mollusks and insects of the family Carabidae were selected for the soil ecotoxicological assays standardized by the main international regulatory agencies (Table 1.1). Those agencies include the American Society for Testing and Materials (ASTM), Environment Canada, the International Organization for

Table 1.1 Summary of the available standard invertebrate species for soil ecotoxicological assays. Table derived from C. A. M. Van Gestel, Soil ecotoxicology: State of the art and future directions, *ZooKeys*, 2012, **176**, 275–296. Copyright 2012 C. A. M. Van Gestel. This was published under the Creative Commons Attribution License 3.0 (CC-BY) (https://creativecommons.org/licenses/by/3.0/legalcode).

Group	Test organism	Species	Endpoint	Test type	Guideline/Reference
Oligoachaetes	Earthworms	*Eisenia andrei*	Survival; growth	Lab. toxicity test	OECD 207;[26] ISO 11268-1;[40] EPA 712-C-016;[41] EPS 1/RM/43[42]
		Eisenia fetida	Reproduction; growth	Lab. toxicity test	ISO 11268-2;[24] EPS 1/RM/43;[42] OECD 222[43]
		Lumbricus terrestris	Avoidance	Lab. behaviour test	ISO 17512-1;[30] EPS 1/RM/43[42]
			Bioaccumulation	Lab. toxicity test	ASTM E1676-12[44]
		Different species	Species diversity and abundance	Field test	ISO 11268-3[45]
	Enchytraeids	*Enchytraeus albidus*	Survival; reproduction	Lab. toxicity test	OECD 220;[46] ISO 16387[47]
		Other *Enchytraeus* spp.	Bioaccumulation	Lab. toxicity test	ASTM E1676-12[44]
Arthropods	Collembolans	*Folsomia candida* *Folsomia fimetaria*	Survival; reproduction	Lab. toxicity test	EPS 1/RM/47;[48] OECD 232;[49] ISO 11267[50]
		Orthonychiurus folsomi *Proisotoma minuta*	Avoidance	Lab. behaviour test	ISO 17512-2[31]
	Mites	*Hypoaspis aculeifer*	Survival; reproduction	Lab. toxicity test	OECD 226[51]
	Carabid insects	*Oxythyrea funesta*	Survival	Lab. toxicity test	ISO 20963[52]
Nematodes	Nematodes	*Caenorhabditis elegans*	Survival	Lab. toxicity test	ASTM E2172-01[53]
Mollusks	Snails	*Helix aspersa*	Survival; growth	Lab. toxicity test	ISO 15952[54]

Standardization (ISO), the Organisation for Economic Co-operation and Development (OECD) and the US Environmental Protection Agency (EPA).

The current number of available species for the standard laboratory ecotoxicological assays (Table 1.1) is considered too low, and demonstrates an under-representation of the diversity of invertebrates inhabiting soils in natural ecosystems or even when compared to the diversity of invertebrate fauna in soils from the agro-ecosystems. The sub-representation is even greater when comparing the number of arthropods with the number of earthworm species selected for the standard tests.[15] The species richness of earthworms in terrestrial ecosystems is generally much smaller than the richness of arthropod species, but these oligochaetes are represented by at least five test species in standard toxicity assays (*Eisenia andrei*, *Eisenia fetida*, *Lumbricus terrestris*, *Enchytraeus albidus*, *Enchytraeus crypticus*—Table 1.1), in addition to a field test with different earthworm species.[45] On the other hand, arthropods comprise about 80% of all soil invertebrate animals and are only represented by the springtail species *Folsomia candida*, *Folsomia fimetaria*, *Orthonychiurus folsomi* and *Proisotoma minuta*, and by the species *Hypoaspis aculeifer* (predatory mites) and *Oxythyrea funesta* (Carabid insect).

Within the limited number of species available for standardized toxicity tests (Table 1.1), only *E. andrei*, *E. fetida*, *F. candida*, *E. albidus* and *H. aculeifer* (Figure 1.1) have been routinely used in batteries of ecotoxicological assays to assess the ecological risk of substances for the soil fauna. It is also possible to conclude that the representativeness of soil invertebrates is further reduced in a higher degree than is supposed by the recent reviews on this subject.[11,15,17,22] According to a number of studies,[55–58] this poor representation of the soil fauna in ecotoxicological testing is, in great part, a consequence of the stringent requirements for the standardization of the assays, which, in general, choose the species based on rigid parameters, such as:

(a) Physiological: the species must have high sensitivity to soil contaminants, low variability (use preferably species with parthenogenetic

Figure 1.1 Summary of the available standard invertebrate species for soil ecotoxicological assays: (A) *Eisenia andrei*; (B) *Folsomia candida*; (C) *Enchytraeus crypticus*; (D) *Hypoaspis aculeifer*.
Photos reproduced with permission from: (B) C. M. Ribeiro, picture of the species *Folsomia candida*; (A) and (C) C. A. Santos, pictures of the species *Eisenia andre* and *Enchytraeus crypticus*; (D) M. Bianchi, picture of the species *Hypoaspis aculeifer*.

reproduction), high reproduction rates, short generation time, and be tolerant to several culturing substrates and artificial soils.

(b) Functional: they must be abundant and representative of a taxonomic group and, preferably, should belong to functional groups with essential services for the functioning of the terrestrial ecosystems.

(c) Practical: species with well-known biology, easy to identify (by taxonomy based on morphology), cultivate, maintain and synchronize (age and/or size) under laboratory conditions. It is also desirable to choose species with a reasonable number of measurable responses (*e.g.* pollutant concentration in tissues, biological disorders in growth and fertility, or genetic changes).

Methods based on standard invertebrate species, described in the International Standards, as those published by ASTM, Environment Canada, ISO, OECD and EPA (Table 1.1), are essentially designed to simplify the comparison between results of different laboratories (regardless of geographic location), as well as to increase the accuracy of the dose–response relationships obtained between substances and test species. This accuracy is fundamental in order to establish appropriate protective limits of exposure for invertebrates in polluted soils, especially in assays performed to release new molecules (*e.g.* of pesticides) to the market. On the other hand, it should also be admitted that the dependence on results only based on standard species may underestimate the real impact of toxic substances in terrestrial ecosystems, since it is not possible to prevent the impacts (*e.g.* species extinction or the loss of ecosystem services) on the immense diversity of invertebrates existing in the soil.[3,8]

For this reason, it is necessary to continue further studies in order to select standard species for toxicity testing, especially among soil arthropods, to improve the representativeness of these organisms in the ecological risk assessments of pollutants. In this sense, species of the order Isopoda (*Porcellio scaber*, *Oniscus asellus* and *Porcellionides pruinosis*) are strongly recommended for inclusion in standardized ecotoxicological assays.[15] Isopoda species have great potential for standard assays because of their high ecological significance in terrestrial ecosystems. Thus, their typical routes of exposure to soil pollutants (by contact and ingestion) and the interesting characteristics of their life cycle, with endpoints alternative to the traditional ones, are promising. In addition, they have been used in toxicity tests for more than 30 years.[15]

Other activities are also being proposed for the selection of new species in soil toxicity tests. Some authors proposed that enchytraeid species of the *Fridericia* genus (*Fridericia bulbousa* and *Fridericia peregrinabunda*) should be considered as new test species in the list of standardized assays for soil ecotoxicology.[37,39] According to these authors, those species have similar (or higher) sensitivity to some heavy metals and pesticides, when compared to some of the standard soil invertebrates. In addition, the *Fridericia* genus has greater representation in terrestrial ecosystems, because this is the genus in the Enchytraeidae family with the greatest species richness of worldwide distribution.[59]

On the other hand, the possibility of using toxicity assays with non-standard (alternative soil invertebrate) species cannot be dismissed. During the last few decades, following the development of standardized toxicity tests, there was also an increase in the number of ecotoxicological studies using non-standard invertebrate species.[15] In general, the research using alternative species has similar objectives to the standardized assays. These types of assays assess the same endpoints as the standardized assays (*i.e.* survival, reproduction and behavior), however, in view of the characteristics of the selected species.

An advantage of the use of alternative species in terrestrial ecotoxicological tests is the higher representation of the local diversity of soil invertebrates. Using species from a particular biome will help to increase the accuracy of the responses for the local fauna, when simulating the impacts in a specific natural ecosystem. Springtails, such as *Onychiurus armatus*, *Protaphorura quadriocellata*, *Orchesella cincta*, *Tullbergia granulata*,[60] *Proisotoma minuta*[61] and *Isotoma* viridis,[62] are used as bio-indicators of soil pollution and are examples of alternative species to increase the ecological relevance of risk analyses of substances in terrestrial ecosystems. The main standard species of springtail (*F. candida*) is recommended in standard assays,[31,42,49,50,55] but has limited ecological relevance, because of its absence in many natural and agricultural habitats.[63]

Moreover, studies based on alternative species have not received the proper attention for several reasons, such as those enumerated below:

I. Many of the selected alternative species are still unknown for most of the researchers working with the soil matrix and, therefore, their use is unusual.
II. These species are native to specific biomes and are not easily found (and identified) in places in which most of the researchers are interested in (they have low ecological relevance in other ecosystems, different from those of origin).
III. The organisms do not have a well-known biology (or it is even unknown) and/or are not suitable for the traditional methods of cultivation under laboratory conditions.
IV. Some species have very long life cycles, which makes it difficult to assess certain parameters, such as reproduction, effects on longevity and heritable genetic damage.

The next section of this chapter will show examples of the use of alternative species in soil ecotoxicology. Only Earthworms, Collembolans, Enchytraeids, Isopods and a few other taxonomic groups will be considered in the following discussion because of their recognized potential for ecotoxicological assays.[12,14]

1.3 Key Groups of Invertebrates for Soil Ecotoxicological Testing

1.3.1 Earthworms

Earthworms are the most frequently used organisms in standardized soil ecotoxicity tests around the world, and even among non-standard species they are the most used. The reasons are related to their ecological importance and the ease of conducting these tests. Earthworms are considered ecosystem engineers in soil because of their key role in soil structure and biological activity, *e.g.*, by building biopores, transferring organic material from the surface to deeper layers, producing humus, improving microflora, micro and mesofauna activity, and increasing plant growth.[8,64,65]

One of the weaknesses of using *Eisenia* spp. (species recommended by standardized guidelines) is the fact that *Eisenia andrei* and *Eisenia fetida* are epigeic species (litter dwelling), *i.e.*, they live and feed only on the surface. Such ecological traits may not represent what happens to other groups of Oligochaetes in soils, *e.g.*, the anecic and endogeic species, which are soil dwellers.[66] Other questions are with regard to their sensitivity to contaminants and representativeness of exposure conditions to different soils and climatic scenarios.[25]

Efforts have been made to identify potentially useful species for different ecozones,[25,67,68] *e.g.*, the earthworm species *Lumbricus rubellus*, *Dendrobaena octaedra* and *Dendrodrilus rubidus* were selected as the most promising candidates for Canadian boreal forest ecosystems.[69] In subantarctic conditions, the toxicity of diesel-contaminated soils was evaluated using the subantarctic earthworm *Microscolex macquariensis* in lethality, avoidance and 2 week reproduction tests. These tests were carried out at 8 °C, a realistic condition for the region. The species reproduced in laboratory tests, but the authors reinforced the need for more studies about its life cycle.[70]

Among the non-standard species studied in ecotoxicity tests, *Pontoscolex corethrurus* Müller (Figure 1.2A) is an endogeic species that shows a wide tolerance to environmental variations, living in many different habitats and soil types throughout the tropics and sub-tropics. Ecotoxicity tests were carried out using the existing guidelines for avoidance behavior and lethality tests, and this species showed similar sensitivity to the standard species *E. andrei* to the pesticides carbendazim, carbofuran and glyphosate.[71] These results could indicate that *E. andrei* is sensitive enough to represent the populations of this autochthonous species. However, this should not be generalized, considering other non-standard species or even other contaminants. *P. corethrurus* was more sensitive to the fungicide carbendazim, but less sensitive to the insecticide lambda-cyhalothrin, when compared with the standard test species *E. fetida* in lethality tests under tropical conditions.[25] *Metaphire posthuma* is an Indian species widely distributed in various states of India and other Asian countries that is well adapted to burrowing. This species was more sensitive to the pesticides carbaryl,

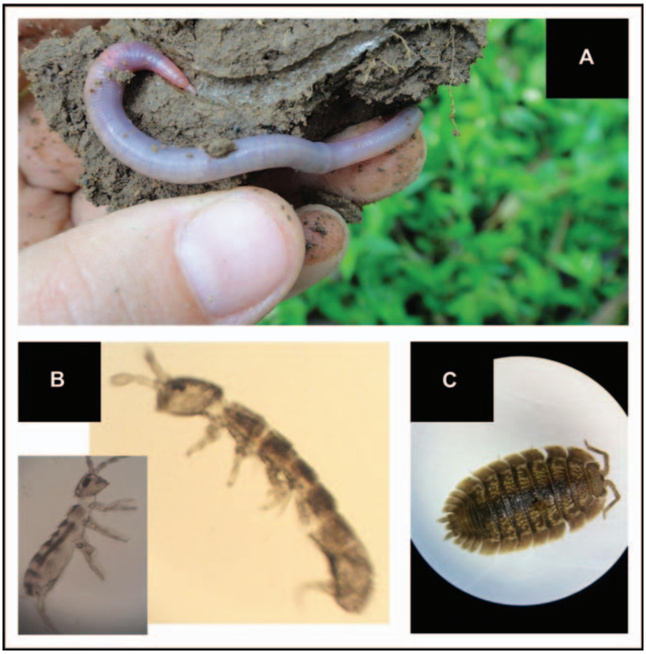

Figure 1.2 Examples of alternative species of soil invertebrates for the standard laboratory toxicity assays: (A) *Pontoscolex corethrurus*; (B) *Proisotoma minuta*; (C) *Porcellio dilatatus*.
Photos reproduced with permission from: (A) M. L. C. Bartz, picture of the species *Pontoscolex corethrurus*; (B) A. Buch picture of the species *Proisotoma minuta*; (C) L. P. Crescencio, picture of the species *Porcellio dilatatus*.

carbofuran, cypermethrin and fenvalerate than *E. andrei* in lethality tests.[72] *Octolasion tyrtaeum*, a common species in many Argentine agricultural ecosystems, was more sensitive than *E. andrei* to glyphosate, when testing adult lethality and variation of biomass.[73] Accumulation and toxicity of metals (Cu, Cd, Ni, and Zn) were studied using *Lumbricus rubellus* (epigeic), *Aporrectodea longa* (anecic), and *Eisenia fetida* (ultra-epigeic) with 28 days of exposure in two soils; under these circumstances, *L. rubellus* was the most sensitive species.[66] In ecotoxicological assessment of imidacloprid, *E. fetida* responded with significant avoidance behavior in all tested concentrations, while *L. terrestris* and *A. caliginosa* did not avoid the contaminated soil.[74] Differences in sensitivity or in bioaccumulation rates among species can be related to differences in ecological strategies (and, consequently, differences in exposure); to physiological mechanisms of detoxification and elimination;[66,75] or to differences in average body mass and surface/volume ratio.[74] Even a hormesis effect was detected in *Lampitto mauritii* for low concentrations of methyl parathion.[76] In general, uncertainty remains regarding the sensitivities of non-standard species in comparison to standard species.

The main challenges to using native earthworm species are the rearing of these organisms in the laboratory.[73] This is one of the reasons why reproduction tests were rarely conducted with non-standard species. The published works with native species reported field collection and laboratory acclimatization of earthworms before the tests, or reported buying earthworms in fishery stores. However, laboratory cultures are important to ensure the quality of test organisms, allowing synchronization of the cultures and avoiding exposure to contamination sources and abiotic stress.[77] Furthermore, field collections can be limited by seasonality in earthworm abundance or risks of confounding species. The difficulty is that some species have a long life cycle and low reproduction rates in the laboratory, *e.g. P. corethrurus*, whose life cycle was completed in about one year in laboratory studies.[78]

Still another consideration is that laboratory studies with non-standard species require knowledge of the biology and ecology of these species, once laboratory cultures and tests must be kept at optimal levels to avoid the influence of such interferences on the evaluated endpoints.[77] Adaptations of the existing guidelines have been made mainly regarding the duration of the reproduction tests, temperature, and amount of soil per replicate, in accordance with the ecological characteristics of the species.[25,70,76] Among the critical factors are temperature, pH, humidity and food quality. Cattle and horse manure are frequently recommended and have been successful for laboratory cultures. Other food sources cited are fresh leaves and litter, depending on the ecological characteristics of each species. The best culture medium for the epigeic species *Amynthas cortices* Kinberg includes sawdust, and this may be related to the presence of certain enzymes that help this species to degrade cellulosic compounds;[79] it was recommended to add mixtures of high and low quality organic residues in order to maintain successful field populations of endogeic and epiendogeic species in laboratory. Much work still must be done to increase the knowledge on the biology and ecology of the native species, their niches and needs, making it possible to select a larger set of species for the ecotoxicity tests.[80]

A new simple behavior test was proposed using *Lumbricus terrestris*, a common epi-anecic species, based on earthworm bioturbation and cast production.[81] Cast production was influenced by several factors, such as pesticide contamination[81] and soil quality.[82] Cast production rates were evaluated with *P. corethrurus* exposed to metal-contaminated soils and it was found to be a sensitive endpoint.[83]

Another interesting contribution to the test array is an alternative arrangement of avoidance tests, comprising two layers of soil, where the vertical burrowing behavior of *Allolobophora chlorotica* was studied when carbendazim contaminated soil was added to the surface of an unamended soil, containing the earthworms, and when the earthworms were laid on the surface of contaminated soil.[84] Earthworms significantly altered their burrowing behaviour to avoid carbendazim, but when they were added to an upper layer of carbendazim-contaminated soil, they remained in this layer, probably because of neuronal impairment.[85]

In general, the studies reinforce the importance of using a multiple selection of species in ecotoxicology, considering different ecological strategies and including relevant species, aiming at predicting harmful environmental effects more accurately. Furthermore, considering the ecological requirements of important terrestrial ecotoxicological test species, most of the standard species are applicable to a wide range of natural soils, while for some "extreme" soils (*e.g.*, very acidic forest soils) alternative test species will be required.[58,68]

1.3.2 Collembolans

Collembolans are active and abundant under most environmental conditions and their activities usually mobilise C and N.[86] They are involved in complex trophic relationships in soil and are an important prey group for generalist arthropod predators in agro-ecosystems.[87,88] In opposition to the recognition of the potential value of this group as bio-indicators of soil quality, basic information about the occurrence and ecology of species is still largely unknown.[89]

Defining food preferences and optimal abiotic conditions is important to guarantee successful breeding in the laboratory. In a food choice test performed with the non-standard collembolan species *Protaphorura fimata* and *Heteromurus nitidus* with baker's yeast, *Saccharomyces cerevisiae*, and unicellular green algae, *Pseudokirchneriella subcapitata*, as food choices, both species preferred yeast.[88] However, different species can feed on fungi, bacteria, protozoa, algae, enchytraeids, nematodes, invertebrate remains and plant tissue.[86,90]

Temperature, moisture and pH seem to be the most important abiotic factors. Besides that, biotic interactions play a key role in the environment, especially related to microbial activity and competition with other faunal species. In laboratory cultures, contamination with predatory mites is a common problem and should be avoided by checking the food quality and the cleaning of the culture environment (recipients, room, and entomological aspirator).

The OECD guidelines[49] present a list of alternative species to be used in reproduction tests, when some prerequisites are attended to, such as: *Proisotoma minuta* (Figure 1.2B), *Isotoma viridis*, *Isotoma anglicana*, *Orchesella cincta*, *Sinella curviseta*, *Paronychiurus kimi*, *Orthonychiurus folsomi*, *Mesaphorura macrochaeta*. Among the prerequisites are: the unequivocal taxonomic identification, knowledge about the life cycles of organisms before the test, and optimal conditions for the species.

Non-standard species have been collected from a range of terrestrial habitats using a variety of techniques, such as suction sampling from vegetation, pitfall traps, and extraction from soils by flotation on water or by the use of Tullgren funnels. Usually the species are cultivated on the same mixture of Plaster of Paris and powdered activated charcoal recommended by the guidelines for *F. candida*.[91]

However, establishment of laboratory cultures of Collembola collected from the field can be a difficult task. Some species of Australian Collembola were collected but did not produce viable cultures in the laboratory.[61] *Sminthurides* sp. and *Entomobrya* sp. collected from forest soils in Brazil showed low adaptation to laboratory conditions.[91] Species with characteristics such as small size, low reproduction rates or more conspicuous colour can be a challenge for use in ecotoxicity tests. *Sinella communis* was shown to be a suitable species for toxicological testing in Australia, being easy to count and being more sensitive to a range of toxicants than *F. candida*.[61] *Folsomia nivalis* was used in a battery to assess boreal forest soils,[68] and was found to be more sensitive than the standard species *F. candida* to hydrocarbon-impacted soils.[69]

Laboratory avoidance tests were conducted with five collembolan species (*Isotoma anglicana*, *Heteromurus nitidus*, *Lepidocyrtus violaceus*, *Folsomia candida*, *Onychiurus armatus*) towards the herbicide Betanal (active ingredient: phenmedipham) in soil.[92] Sensitivity was dose-dependent and species-specific, with *O. armatus* being the most sensitive species. At higher concentrations (near the calculated LC_{50} value), however, a higher number of organisms were found in contaminated soil, which can be a possible narcotic effect of this substance.

Soil characteristics can alter the toxicity of contaminants and also act as stressors themselves, as observed during the reproduction of *Paronychiurus kimi* in cadmium-contaminated artificial soil.[93] However, papers are scarce about the sensitivity of non-standard species to contaminants and about the influence of soil properties.

In conclusion, many advances are needed to increase the basic knowledge about non-standard collembolan species, and their ecology, performance under laboratory conditions, and sensitivity to contaminants and soil properties.

1.3.3 Enchytraeids

Enchytraeids are small oligochaete worms, generally considered to be saprovorous and microbivorous, stimulating microbiological activity in soil through grazing and dispersion of spores.[94]

Some species are easy to rear and are cultivated as food for fishes by some ornamental fish breeders. However, basic ecological studies with non-standard species of enchytraeids remain poorly understood, which is caused by a lack of taxonomists, unfamiliarity with collection methods or even ignorance about this group.[95]

ISO guidelines[47] bring a list of potential species to be used in enchytraeidae reproduction test: *Enchytraeus crypticus* Westheide & Graefe, 1992, *Enchytraeus buchholzi* Vejdovsky, 1879, *Enchytraeus luxuriosus* Schmelz & Collado, *Enchytraeus bulbosus* Nielsen & Christensen, 1963. However, the most important criteria for species selection, besides basic knowledge about culture requirements, is the ecological relevance and sensitivity to contaminants in comparison to the standard species *Enchytraeus albidus*.

Besides the standardized reproduction test, avoidance tests were proposed for the standard species *E. albidus*;[96] however, because of the lower sensitivity and higher variability, the enchytraeid avoidance test was not recommended for risk assessment purposes.[97]

Among the non-standard species, *E. crypticus* has been successfully applied in ecotoxicity tests around the world and has the advantage of good performance and speed of reproduction.[38] This species is sensitive to a range of contaminants and it can be used in risk assessment of contaminated sites.[98–100] Alternative endpoints using embryotoxicity tests were proposed for this species.[101]

Other species are mentioned in the literature, especially in studies with metal contamination: *Fridericia bulbosa* in lethality tests, *Enchytraeus doerjes* and *Enchytraeus bigeminus* in reproduction tests.[37,39,102–105]

Other aspects, such as the influence of soil properties on performance of enchytraeids and their interaction with soil contaminants, should be better understood.[106]

1.3.4 Isopods

Terrestrial isopods are saprophagous soil organisms that have a key role in litter fragmentation. The availability of studies about soil isopods, their ecological relevance and the ease of manipulating them make this group one of the most promising to be included in standardized protocols.[15,34]

No standardized species or guidelines exist at this moment for isopod tests. Among the challenges for their standardization are the high variability among individuals, probably because of sexual reproduction and the long timescale for reproduction in comparison to other invertebrate species, low reproduction rates, different sensitivities between males and females, and lack of knowledge about the basic ecology and life cycles of the species. Information on the methods to maintain and rear isopods in the laboratory is available for some species.[107–109] The main factors influencing isopods in culture include humidity, temperature, pH, and food quality.[110–112]

Porcellio scaber is one of the most used isopod species in ecotoxicity tests. Other species, such as *Porcellionides pruinosus, Porcellio laevis, Porcellio dilatatus* (Figure 1.2C), *Armadillidium vulgare*, *Cubaris murina* and *Oniscus asellus*, are suitable for ecotoxicity tests for a range of contaminants. Avoidance behavior tests were proposed for isopod species[113] and have been found to represent a more sensitive endpoint than lethality or sublethal endpoints,[114] suitable for screening of contaminated sites.[115] However, their tendency to aggregate could be a challenge for avoidance tests, because this behaviour might lead them to choose sub-optimal conditions.[114]

Lethality is considered a low sensitive endpoint because isopods can decrease food consumption (= intake) and tolerate high concentrations of contaminants in the environment.[116,117] Biomass loss is one promising endpoint for ecotoxicity tests because of its great sensitivity and ecological relevance,[118] since weight loss is related to lower reproduction and consequently lower success of populations in the environment.

Reproduction tests are proposed with the exposure of non-gravid females,[119] as well as with exposure of truly gravid females.[120] Other endpoints have been proposed, such as food consumption determined by faecal production rates,[121] bioaccumulation,[122] feeding behaviour.[123–125]

Enzymatic biomarkers have been evaluated in tests with isopods to help to understand chemical stress modes of action, but some authors have shown high levels of inter- and intra-specific variability.[126,127] Cell membrane damage by direct contact or by lipid peroxidation in *P. scaber* is caused by the ingestion of titanium dioxide nanoparticles.[128] Effects of endocrine-disrupting compounds on the molting regime, growth and protein expression in different organs of isopods have been studied.[129,130]

In general, a substantial body of experience should be obtained with biomarkers in isopods in order to facilitate their application.[131] Furthermore, improvements are necessary to optimize the already developed ecotoxicity tests, aiming at proposing a guideline with this important group for soil ecosystems.

1.3.5 Others

Studies with soil invertebrates besides those cited above are scarce. Among the groups of non-standardized species that have been used are mites and beetles.

Mites are abundant soil organisms involved in decomposition of organic matter, in nutrient cycling, trophic structure and dynamics in soil. Among mites, *Oppia nitens* is a good candidate species for a standardized test design, with adult survival easily assessed in a relatively simple design. A long-term reproduction test with *O. nitens* will require the use of a synchronized population and, on occasion, organic matter amendment when testing soils with low organic matter content.[132]

Beetles of the family Scarabaeidae are important organisms that promote the decomposition of the dung pat, destroying possible habitats for cattle parasites, allowing the release of nutrients into the soil and for plant growth, and acting as food sources for insectivorous birds and mammals.[133,134] That is why it is so important to study the effect of veterinary products on these species. A test with the temperate dung beetle *Aphodius constans* was developed[135] evaluating the survival of beetle larvae in 3 week duration tests with fresh dung as the substrate. The larvae were exposed to four veterinary parasitical pharmaceuticals (ivermectin, moxidectin, dicyclanil, and praziquantel) representing different treatment regimes, modes of action, and effect levels. This test was recommended for standardization in an international ring test for risk assessment of veterinary pharmaceuticals.

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