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# Methodological Considerations in the Study of Earthworms in Forest Ecosystems

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Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/67769>

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## Abstract

Decades of studies have shown that soil macrofauna, especially earthworms, play dominant engineering roles in soils, affecting physical, chemical, and biological components of ecosystems. Quantifying these effects would allow crucial improvement in biogeochemical budgets and modeling, predicting response of land use and disturbance, and could be applied to bioremediation efforts. Effective methods of manipulating earthworm communities in the field are needed to accompany laboratory microcosm studies to calculate their net function in natural systems and to isolate specific mechanisms. This chapter reviews laboratory and field methods for enumerating and manipulating earthworm populations, as well as approaches toward quantifying their influences on soil processes and biogeochemical cycling.

**Keywords:** earthworms, lumbricids, soil fauna, ecosystem engineer, soil methods, faunal manipulations, arthropod exclusions, soil microcosms, electroshock

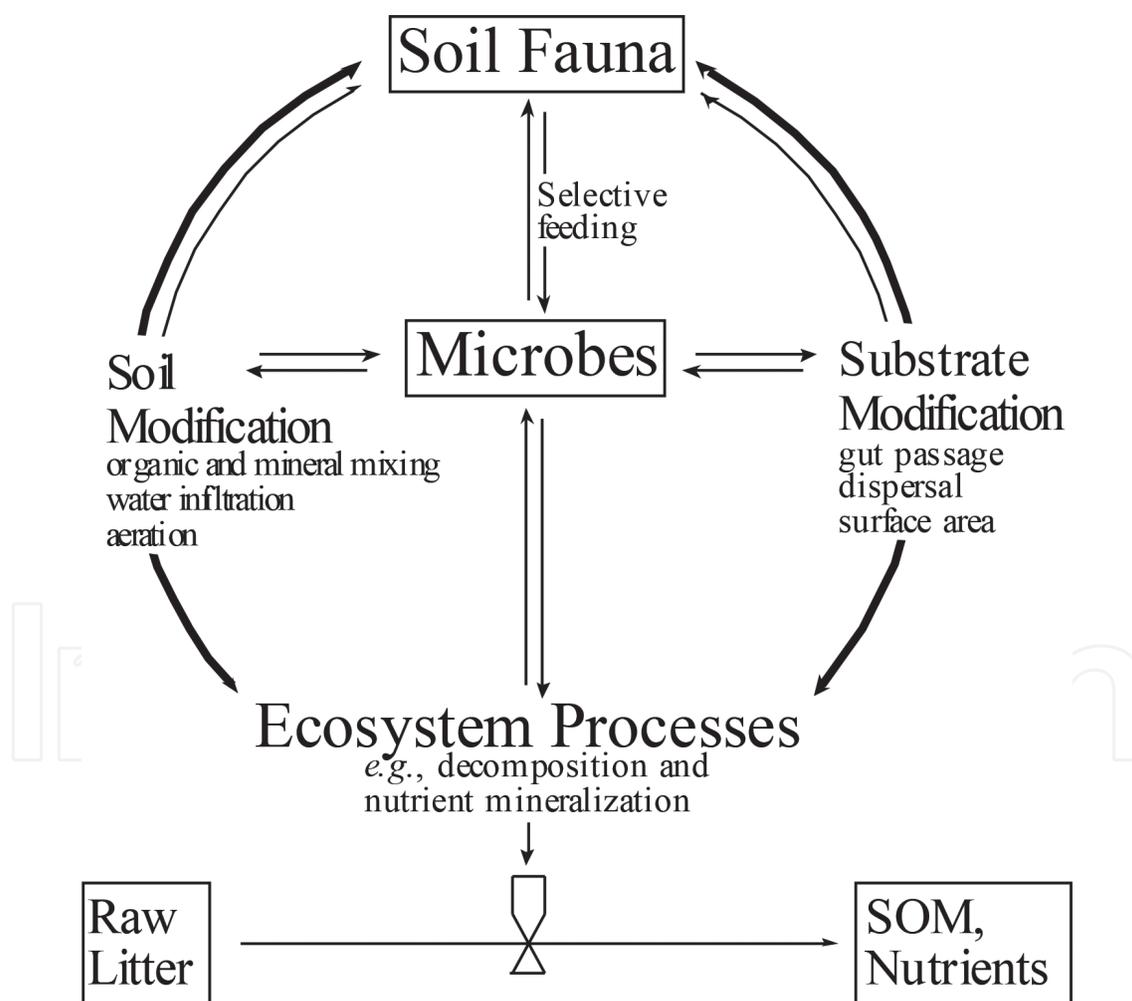
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## 1. Introduction

The impact of earthworms on soil dynamics can be defined as changes in physical characteristics, microbial activity, and nutrient chemical conditions. However, these processes are interconnected to an extent while attempting to separate them can prove difficult. Physical effects of earthworms can be attributed to their feeding and burrowing behavior. Initial contact of earthworms with litter detritus or crop residue is often by comminution or fragmentation, which in effect reduces the size of both organic and mineral particles [1]. This increases the surface area for soil fauna and microbes to act upon. Individual soil

microbes are limited by the inability to relocate and actively seek new substrates, thus their effect on the actual rate of chemical transformations may be more regulated by mechanisms that bring them in contact with new organic substrates than the total amount of substrate available [2], **Figure 1**.

While the soil microbial biomass is directly responsible for the majority of biogeochemical cycling and nutrient mineralization in soils (at least 90%), often the players that link such activities to higher spatial scales through organization and activation, such as roots and soil invertebrates, are largely ignored [2]. Earthworm casts are biogenic structures produced as a result of gut passage, mixing organic and mineral soils. The consequences of this aggregate formation can be physical in nature, including increased drainage and moisture loading capacity [1]. Both permanent and evanescent burrows, sometimes reinforced with protein-based mucus, can promote soil porosity and thus aeration, reducing anaerobic



**Figure 1.** Conceptual model illustrating direct and indirect pathways of interactions between soil fauna, microbes, soil physical properties, substrate, and ecosystem processes [44].

conditions and increasing gaseous exchange, and thus promoting microbial activity, as well as infiltration [1, 3].

Earthworm populations differ significantly in terms of numbers, biomass, and diversity across the regions in which they are found. Population sizes are often determined by readily available organic matter, as well as soil type, pH, moisture capacity, precipitation, and ambient temperatures [4]. In most soils, earthworm biomass exceeds that of all other soil invertebrates [1].

There have been several efforts to standardize earthworm sampling across biomes [5]. The basic approach to identify the influences and mechanisms by which earthworms influence soil systems and detrital food webs is most often pursued via controlled experiments comparing earthworm containing soils to those void of them (controls). Most often this is achieved by expelling target fauna from a sample of soil. This can be no simple task working in the “black box” that is the domain of soils all the while striving for minimal disturbance in important soil properties such as structure, pedology, and faunal composition.

This chapter will serve as a review of the methodologies applied in past experiments, field work, and modeling efforts involving the influence of earthworms in forest soils. We will remind soil researchers of the plethora of challenges faced in soil research and argue that no singular method or tool is a panacea to the difficulties that may arise. This review provides a perspective into faunal experiments and a toolbox of techniques and approaches to evaluate and quantify the influences of lumbricids in terrestrial environments.

## **2. Microcosm laboratory and mesocosm field experiments**

A traditional, effective method in studying the myriad of influences of earthworms on soil ecosystems is the laboratory microcosm. The concept of the microcosm is to recreate a miniaturized version of the ecosystem under study in controlled lab settings in order to control all variables possible that are not those under question. Most commonly for soil and earthworm studies these microcosms consist of a plastic PVC cylinder ranging between 6 and 16 cm in diameter and 15–50 cm deep, but other materials such as plexiglass containers [6] or glass jars with perforated lids [7, 8] have been utilized to incubate between 75 and 150 g of soil substrates. These effectively act as experimental soil cores. Relatively larger controlled soil environs, known as mesocosms, can be placed in the field to subject the closed system to more natural climate conditions. These can be made of buckets with perforated bottoms (25 cm diameter, 8 kg soil [9]) or clay pots (4.5 L), which have the ability to maintain a desired moisture regime [10].

Usually a metal, plastic, nylon [11], or fiberglass [12] mesh of 1 mm [13] to 2 mm [6] is placed on the top and/or bottom of containers to prevent escape (or colonization in mesocosms) of earthworms as well as retain soil and moisture. For microcosms, these soil cores can be taken from the field intact [14, 15] by hammering the cylinder into the ground, removing, and returning to the lab for observation. Alternatively, microcosms can be filled with homogenized soils

(often also gathered from the field) after being passed through a 2.5–4 mm sieve [12, 16–18]; however, this may destroy preexisting soil aggregate structure. Other substrates have been used in earthworm microcosms such as pig manure [8].

Soils used to fill microcosms may be gathered from a range of ecosystems in which earthworms may be found: tropical, temperate, agroecosystem, grassland/savannah, or forest. Recognizing the importance of pedology, Lachnicht et al. [19] sampled both the O- and A-horizon to incorporate into their mesocosms from the tropical forest soil they aimed to simulate. Alternatively, open bottomed microcosms can be returned to the field after manipulation [9, 11, 20, 21] and buried to retain field climate conditions in “field microcosms,” more akin to the mesocosm approach.

In laboratory microcosms, a polyethylene film can be placed on the bottom of the cores to prevent leaching [14]. If leachate chemistry is under investigation, ceramic lysimeters can be installed and drained under semi natural conditions (–200 to –400 hPa [18]). Alternatively, if soil moisture chemistry is under study, microcosms can be capped with ceramic plates and atmospheric pressure reduced (–0.5 atm) to collect soil solution otherwise bound by capillary forces [17, 22]. Costello and Lamberti [23] use passive soil water percolation and collection of leachate to add to a stream mesocosm to assess periphyton growth in an elaborate simulated riparian study and effect of soil fauna on stream inputs.

A variant of the mesocosm experiment is the greenhouse pot or bag experiment, which involves established vegetation in a contained soil pedon or core; often exploited in investigating earthworm effects on plant growth or allocation [24, 25]. Similarly, plants can be added to the mesocosms previously described [13].

Most microcosm/mesocosm studies involve the inoculation of the substrate with the desired earthworm species (or functional group), community composition, density, and biomass for the study, often with multiple treatments. Climate conditions can be easily controlled in laboratory or greenhouse settings in addition to avoiding predation on earthworms. A common practice is to allow earthworms to void gut contents for 36–48 hours before being added to microcosms [15, 19, 25] to prohibit influences from outside origins or substrate, especially in studies involving isotopes.

Stable isotope ( $^{15}\text{N}$  and/or  $^{13}\text{C}$ ) labeled crop residue can be applied to mesocosms to track the assimilation of substrate and to discern from soil organic matter in earthworm tissues [7, 19, 26–29]. Microcosm experiments can range from 72 hours [8] to 12 days [7] to 16 weeks [12], to 120 days for the plant-pot experiments [24]. However, there is debate over the duration of microcosm experiments. Whalen et al. [26] argue less than a week for studies concerning excretion rates using  $^{15}\text{N}$  to avoid reingestion and help discern between structurally incorporated N and excretions. Artificial earthworm burrows have even been created to compare abiotic and biotic influences of the burrows created by anecic species [12, 30].

To identify and isolate the effects solely of the internal gastrointestinal ecology of earthworms, Barois and Lavelle [31] dissected *Pontoscolex corethrurus* individuals sampled from an agricultural field, observing and comparing soil in the anterior, middle, and posterior thirds of the gut-deducing changes in physicochemical and respiration properties of soil during gut

transit. In a laboratory setup, Horn et al. [32] sedated earthworms with 40% ethanol, laying them horizontally and partially embedded in 1.5% agarose to implant microsensors. They then could study the internal earthworm gut environment to analyze the activation of nitrous oxide-producing bacteria.

Various methods have been applied to manipulate preexisting earthworm communities in the soils used as substrates in the micro/mesocosm experiments before controlled inoculations are performed. Many of these are not possible in field experiments. Homogenized or sieved soils are usually depleted of earthworms and their cocoons via hand-sorting [14, 25]. However, with intact cores where preservation of soil structure is desired, different methods may be needed. Fonte et al. [11] use a modified electroshock technique (discussed here later, mainly for field experiments) for earthworm extraction. Willems et al. [14] use both the modified electroshock method and heat of 40°C for 48 hours to kill any remaining individuals or cocoons in intact cores. Defaunation by Butenschoen et al. [18] was achieved by freezing soil at -28°C for 2 weeks, followed by a week preincubation for microbial recovery. Similarly, after sieving (5 mm), Lenoir et al. [33] froze soil samples at -20°C for 3 days in an attempt to kill most meso- and macrofauna, noting that many microfauna (nematodes, rotifers, protozoans, tardigrades, and microarthropod eggs) survived. Huang et al. [20] froze intact PVC cores taken from the field (-30°C) for 48 hours to kill earthworms. After sieving, Alpehi et al. [34] subjected soils for used in their mesocosms to two chloroform fumigation cycles, reestablishing microbiota with unfumigated soil slurries. Postma-Blaauw et al. [28] irradiated soil with  $\gamma$ -radiation (25 kGy) to sterilize, previous to reinoculating with microbiota and adding to mesocosms. Alternatively, soil can be sterilized with methyl bromide [34]. Soils can also be dried in the shade for a period of time [24] or autoclaved [25] to eliminate earthworm cocoons. In all of the above methods, effects on nontarget organisms must be considered and reinoculation of microbiota is often necessary. Soil organisms are extraordinarily diverse, spanning the three domains; the loss of key species or functional groups may affect interacting species and thus potentially large changes in the soil ecosystem processes. Additionally, the unintended contribution of dead earthworms or other fauna to soil organic matter must be considered in these methods that fail to remove fauna and kill them in place.

Barot et al. [35] criticize the use of micro- and mesocosms, as soils are usually homogenized—disrupting soil horizons, and partially defaunated. Carpenter [36] reviews microcosm experiments, arguing the usefulness and applicability of this approach; listing rapid results at relatively low costs, ease of replication and repetition, and ensuing statistical advantages. This allows enhanced power over experimental controls, testing specific mechanism hypotheses, and deriving rate estimates. Indeed high level of replication and control over abiotic factors can be desirable in variable heterogeneous medium such as soils. However, Carpenter [36] cautions of the danger of losing context and appropriate scale both spatially and temporally, leading to possible distortions in community and ecosystem considerations. In general, microcosms can be an indirect way to study ecology: “Without the context of appropriately scaled field studies, microcosm experiments become irrelevant and diversionary” [36]. Taking a computer model approach, Barot et al. [35] expand microcosm studies to predict long-term earthworm effects on primary production; however, they emphasize field studies must follow to confirm model-based conclusions.

### 3. Field manipulations

While laboratory experiments can be useful for examining specific mechanisms and chemical pathways, in lieu of the discussion above, it is apparent that field experiments are necessary to confer results before large-scale extrapolation. Different techniques can be utilized to remove or expel earthworms from manipulated plots. It must be recognized that no one method is likely to completely exclude earthworms, especially in longer-term studies, thus treatments can often be considered as reductions rather than eliminations or exclusions. To maintain earthworm treatments, investigators regularly install physical barriers to define experimental soil plots. Parmelee et al. [37] installed plexiglass enclosures to 25 cm depth and 30 cm above ground in their agroecosystem field experiment. A common practice is to bury plastic sheets (PVC) to 45–50 cm with 10–15 cm above ground to restrict lateral movements of earthworms in both agroecosystems [38–41] and forests [42]. Alternatively, plastic garden edging (to ~20 cm [23]) in a forest or corrugated plastic to 5 cm depth and 25 cm above ground [43] in an agriculture field can be used; however, this may be an inadequate depth to control all earthworm movement. González et al. [44] noted that the garden liner they used in combination with an aluminum fence (15 cm above ground) allowed both oxygen and water exchange to either side of the barrier; an important detail that other studies fail to address.

#### 3.1. Faunacides

##### 3.1.1. Naphthalene

Naphthalene is a general repellent for arthropod communities and has thus been widely applied to studies seeking fauna contributions to decomposition. However, it is unknown and rarely reported what effects naphthalene has on earthworms. González and Seastedt [45] do report that earthworms were found in plots that had been hand-sorted to 20 cm, lined with weed/garden liner and treated with naphthalene. Heneghan et al. [46] report over 58% reduction in arthropods in their temperate vs. tropical system study. Naphthalene was found not effective in reducing arthropod abundance compared to control plots in a tropical dry forest where numbers were already low [44]. In addition to the lack of specificity and knowledge concerning effectiveness with earthworms, naphthalene is known to affect microbial communities. For example, González et al. [44] found no net change in total microbial biomass, yet the abundance of salicylate mineralizers specifically was enhanced in naphthalene applications, having implications for lignin degradation and increased immobilization of nutrients in the microbial biomass. Work by Blair et al. [47] found nontarget effects of naphthalene where its application in soil-litter mesocosms directly affected both microbial abundance and activity. Additionally, it appeared that microbes utilized naphthalene as a carbon source, illustrated by increased soil respiration rates. Furthermore, naphthalene treatments drove net nitrogen mineralization compared to net immobilization in controls. Based on this brief collection of findings, naphthalene cannot be advised for application for earthworm reduction nor general faunal exclusion, especially when biogeochemical pools and fluxes are concerned.

### 3.1.2. Carbofuran

Carbofuran (or carbofuranadan or carbamate, 2,3-dihydro-2,2-dimethyl 1,7-benzofuranyl methylcarbamate) can be applied to eliminate soil fauna [48], in amounts and frequencies ranging from once at  $0.41 \text{ g m}^{-2}$  in field microcosms [39] to twice over 6 months at  $25 \text{ g m}^{-2}$  in  $4 \text{ m}^2$  field enclosures [37] prior to earthworm inoculation. Alegre et al. [49] report natural elimination of the faunacide after 4 weeks, although questions of lasting nontarget effects and subsequent influences on investigative results remain.

Gilot [39] reports incomplete elimination of earthworms with a single application of carbofuran. While they specifically observed earthworm casts to assess significant differences in earthworm treatments and organic inputs on soil structure, they fail to caution interpretations in the light of nontarget effects of the carbofuran nor do they report earthworm establishment success.

Broadbent and Tomlin [50] report no significant differences in earthworm mean biomass between carbofuran-treated and control plots in an agroecosystem. However, their study found that a broadcast application of carbofuran was more effective than row application. They concluded that in this cultivated field experiment carbofuran application affected the short term, but not long-term community of soil decomposers.

Parmelee et al. [37] applied carbofuran as a vermicide in an agroecosystems field study. They report that initial earthworm reductions were greater in their no-till treatment (79%) compared to conventional till. However, after 286 days both no-till (98%) and conventional till (100%) had greatly reduced earthworm abundance in carbofuran-treated plots compared to controls. Conversely, this study, unlike many others in the past, assessed the nontarget effects of the vermicide treatment. Densities of microarthropod, enchytraeids, nematodes, and bacteria were reduced in at least some of the carbofuran-treated litterbags on some dates. However, these effects neither were consistent over time or till treatments nor reflected in litter decay rates, making them impossible to correct for in final calculations. The authors, therefore, stress that the OM processing rates contributed to earthworms in this study are a potential maximum and may be overestimated due to confounding effects on nontarget biota.

In a review and synthesis of the target and nontarget effects of applied biocides, Ingham [51] reports that in addition to earthworms, carbofuran reduces populations of beetles, weevils, assorted borers, nematodes (of various functional groups), springtails, and *Rhizobium* (at high concentration applications) and can alter fungal dominance 1 year after application. Recognizing that earthworms can play an ecological engineering role yet still remain a single component of the complex hierarchical detritivore food-web, when attempting to tease out the role of earthworms themselves researchers should strive to avoid the nontarget effects of such faunacides as carbofuran.

### 3.2. Passive methods

Relative to application of faunacides, more passive methods for obtaining soil within the sphere of earthworm influence (drilosphere) exist. The burrows of anecic earthworms by

definition open to the soil surface, providing visual evidence allowing investigators to sample burrow soil [52, 53]. Alternatively, the presence of surface-casting species provides investigators with a visual cue to compare drilosphere and nondrilosphere soil by sampling the casts themselves (to compare microfauna of riparian and pasture soils [54]; or to subject to simulated rainfall [55]) or underlying soil [56]. Other visual indicators can be exploited in Northern temperate forests where patches of soil invaded with exotic earthworms contrast greatly with earthworm-free patches where thick organic horizons remain [57, 58]. A quite different passive approach employed by Lavelle et al. [59] is utilizing the difference in size of soil aggregates and the casts of geophagous earthworms by passing soil through a 2 mm mesh sieve to exclude casts. This technique worked in this study looking specifically at casts; however, the effect of earthworms extends beyond the casts themselves. For direct study, fresh casts can be obtained by lightly squeezing on the posterior end of a collected or raised worm [31, 59]. While working in a sensitive area that prohibited addition or extraction of any elements to the soil system, Nuzzo et al. [60] utilized the practice of artificial cover. Similar to herpetofaunal studies, placing “cover boards” made of untreated, rough-cut lumber, on top of the soil and checking them every 2–3 weeks allowed them to estimate community composition, returning individuals after sampling. This method is completely reliant on earthworm activity near the surface (thus biased towards epigeic species) but may be a better indicator of active biomass than total earthworm biomass derived with other methods. A similar passive method, pitfall traps are used for litter and detritus-dwelling mesofauna and have been seen limited application for the collection of epigeic species in Northern temperate forests [61]. While decreased disturbance on the soil system under study is often greatly desired, the aforementioned methods have limited applicability.

### 3.2.1. Litterbags

Litterbags are a commonly applied technique for decomposition studies. Along with the physical-chemical environment, biota, and substrate quality are the driving factors of decomposition [44, 62]. Soil ecologists have utilized litterbags filled with preweighed material and placed in the field to study these factors. Not only can litter of different chemistry be applied and placed in different sites in transplant experiments [63], but also the mesh size of litterbags can vary, limiting which size class fauna (body diameter) can access them. Indeed a functional definition of litter and soil micro-, meso-, and macrofauna is by body diameter (45  $\mu\text{m}$ , 1 and 5 mm, respectively; [64]).

Filley et al. [65] determined the use of 1 mm mesh litterbags was sufficient to exclude all macrofauna in their study including earthworms in northern temperate deciduous forests on a successional spectrum. In a comparison study between Northern deciduous and conifer forests in Colorado, González et al. [63] used litterbags of two different mesh sizes to quantify the effects of different groups of soil fauna on the decay of aspen leaves and lodgepole pine needles. They found litterbags with the small mesh size (1.8  $\times$  1.6 mm) did not inhibit the activities of litter microarthropods but excluded macroarthropod effects on decay. Therefore, by using this technique on comparing sites, they were able to show that both litter microarthropods and the macrofauna are important determinants of decay in the lodgepole pine

forest. Although litter decomposition in these subalpine sites might be influenced differently by various groups of soil and litter fauna [63].

Barajas-Guzmán and Alvarez-Sánchez [66] used litterbags of 1 and 6 mm mesh in a tropical rainforest experiment to assess faunal contribution to decomposition rates; however, the authors do not specify which particular fauna were excluded. In a humid tropical forest in southwestern China, Yang and Chen [67] use litterbags of 2 mm mesh to allow most macro-, meso-, and microfauna, while 0.15 mm mesh was applied to exclude most macro- and mesofauna. In their application of litterbags in a subtropical wet forest canopy trimming experiment, Richardson et al. [68] found that relatively smaller (0.475 mm) compared to larger (1.8 mm) mesh sizes only influenced the abundance and biomass of microarthropods by excluding larger organisms, but not causing major changes in taxonomic composition. Still, in the same experiment, González et al. [69] found negative correlations between mesh size and percent mass loss in litterbags, and between the Margalef index of diversity for the litter arthropods contained in the litterbags and the percent mass loss, suggesting that functional complexity is an important determinant of decay in their forest.

Litterbags are effective in excluding fauna of different size classes, but not specifically earthworms when other fauna in the same size class (ants, termites, millipedes, centipedes) are present. In longer-term experiments, one could presume that the non-epigeic (litter-feeding) species could still obtain access to the substrate on the underside of the litterbag where products of humification have begun to accumulate or fungal hyphae have colonized. However, initial fragmenting and communitation are often the central mechanisms addressed in these studies. González and Seastedt [45] and González et al. [62] use mesh of  $1.8 \times 1.6$  mm, as to not inhibit indirect effects of earthworm casts. Suárez et al. [58] considered possibilities of faunal restriction in their litter decomposition experiment concerning earthworms by using litter boxes instead of bags to eliminate faunal constraints.

Litterbags can be used in conjunction with earthworm exclusion techniques discussed (carbofuran [37]; electroshock [42]; sieving [44]; utilizing mosaic landscape of earthworm-free, and invaded patches [64]). In a combination of the field placed mesocosm and the mesh litterbag for exclusion methods, Cortez et al. [70, 71] buried cylinders horizontally in the soil with two sized mesh treatments. Mesh of 0.5 cm allowed the entry and passage of earthworms, while 0.1 cm mesh prohibited earthworm access, feeding, and influence on the substrate contained inside.

Further criticism of the litterbag technique is the often lack of acknowledgement that some mass loss can be attributed to physical leaching and subsequent transportation, not just direct mineralization [64, 72]. However, this can partially be taken into account by sampling at day 0 of the experiment. Knowledge of the detritivore community present must be applied when considering use and mesh size of litterbags in field decomposition experiments [64].

### 3.2.2. *Earthworm additions*

The addition of earthworms to experimental field plots, similar to that of meso/microcosms, would appear a sound approach to unearth the influences of earthworms, either in plots previously purged of or still containing established populations. Butt [73] reviews earthworm

addition methods for the purpose of bioremediation in the United Kingdom, listing several factors that may lead to unsuccessful establishment in earthworm addition treatments. When extracting individuals using a repulsion technique such as formalin (discussed here later) and then broadcasting on the surface of the soil, survival may be limited by the exposure of the animals to predation and desiccation. The possibility also exists of the extraction method harming the individuals (and thus adversely affecting survivability), being biased to anecic species, and/or the difficulty in transferring cocoons. Some of these potential restrictions can be ameliorated if earthworms are placed beneath the soil surface; however, this will introduce a soil disturbance that may be undesired in many studies considering soil structure and microbial biomass, especially in the short-term. Butt [73] stresses the importance of site history, origin, and life history of earthworm species used. He suggests the ideal method for earthworm inoculation involves a “starter culture” allowed to develop in a bag of soil for a few months so that inoculums include adults, cocoons, and hatchlings. However, his review is in consideration of harsh or disturbed environments that need remediation such as landfills.

After eliminating previously existing populations via electroshock (discussed later), Costello and Lamberti [23] removed surface litter and lightly aerated the upper 5 cm of soil to assist the introduction of earthworms into 0.25 m<sup>2</sup> plots. After a 30-day field incubation, the earthworms were again removed via electroshock, allowing assessment of addition success. They concluded that the endogeic *Aporrectodea caliginosa* (sometimes labelled “endoanecic” [60]) were more successful in establishment after addition than for the anecic *Lumbricus terrestris* and *Lumbricus* spp. juveniles. The removal of litter and disturbance of upper soil may have aided earthworm entry and establishment but may be undesirable in smaller scale studies (temporally or spatially) where soil structure and horizons are considered.

Subler et al. [43] applied earthworm addition treatments (100 m<sup>-1</sup>) to two temperate agroecosystems, comparing them to controls. In this study, earthworms were collected for additions by formalin extraction, thus yielding primary *L. terrestris* individuals. Following the five-month duration of the experiment earthworm abundances were sampled via hand-sorting and formalin (both discussed here later). Despite heavy inoculation numbers, no significant difference in earthworm abundance was found between addition and control treatments at experiment termination. However, while no taxonomic nor functional group information was provided in this study, the addition treatments were found to have significantly fewer “surface-dwelling” species and greater “deep-burrowing” species compared to the control plots. Considering the behavior of anecic species to remove surface organic matter and incorporate it into lower soil horizons in their burrows and these findings, possible competition between anecics and epigeic earthworms is possible.

Similarly, Shuster et al. [74] employed earthworms extracted from nearby no-till cornfield via formalin repulsion in addition treatments and compared to ambient populations. Each spring and fall 100 individuals m<sup>-2</sup> were added to each addition treatment. Epigeic earthworm abundances were reduced in response to additions, possibly due to removal of surface residue by anecics. These findings caused the investigators to question whether earthworm populations ever reached a steady state during their experiment. The authors discuss how added earthworms on top of an existing population could increase burrow and forage activity in the face of limited resources. Furthermore, they suggest much less anecics could be

added to recreate a situation that occurs in natural systems of few anecics in coexistence with other functional groups. They also question the appropriateness of adding anecics to floodplain soil, as they may not be well adapted for the constant flooding and high water tables in these environments. This discussion should lead to caution and concern in the application of earthworm addition treatments in field experiments and in the interpretation of the results derived from these artificially forced communities, especially where OM resources can become competitive.

### 3.2.3. Tagging

Capture-mark-recapture programs have seen wide spread use for terrestrial, aquatic, and volant megafauna for decades, providing vital information on life-span, reproduction, site fidelity, migrations, and habitat use of individuals which can be extrapolated to the population level. While this type of data would be incredibly useful for soil macrofauna, it has not been feasible in the past considering the toxicity of some marking methods, or the size and thus impairment to locomotion of most tags. However, some subcutaneous marker dyes show promise. Recently, nontoxic marker tags made from visible implant elastomers (VIE) with fluorescent properties were applied to earthworm mark-recapture studies with success in *L. terrestris* in a laboratory microcosm [75], and with *P. corethrurus* in field mesocosms in a tropical pasture and wet forest [76]. This method has since been applied in field experiments to study the dispersal of earthworms [77] and shows great promise for spatial and seasonal distribution, age structure, longevity, and range expansion studies.

## 3.3. Physical extraction

Methods for extracting earthworms were first developed to assess annelid population densities and community composition but can equally be applied to field exclusion experiments. These methods can be divided into two categories: physical—those that depend on the physical examination by the researcher within a known volume of excavated soil, and behavioral—those that depend on the behavioral response of the worms to an irritant employed by the researcher, allowing collection at the soil surface [78].

### 3.3.1. Hand-sorting

Coleman et al. [78] give an overview of sorting soils by hand for earthworm sampling. Hand-sorting typically samples a 25 × 25 cm area. While small sample areas increase the fraction of fragmented worms and can be inefficient where population densities are low, larger areas can decrease efficiency purely due to the time required to process the amount of soil. Wet sieving or washing can be applied in addition to detect smaller species and cocoons; or instead of hand-sorting in grassland systems where fibrous roots are very dense [78].

This method is well and broadly applied in studies seeking relationships in environments with variation in earthworm abundance, community structure and diversity alone [79], or in correlation with other factors such as tree species or communities [80, 81], elevational gradients [82], land use [83], or chronosequences of succession or agricultural abandonment [84, 85], or more experimental manipulations such as litter exclusion [86] or litter addition [80].

Hand-sorting or sieving can be undesirable in field experiments as it destroys the pedology and soil texture of the site (thus porosity and hydrology) and likely disturbs many other classes of soil biota. While time and labor consuming, control (or ambient) earthworm treatments could also be sorted without earthworm removal to duplicate the disturbance; however, the resulting soil would not reflect any natural system, even rarely agroecosystems. Zhang et al. [21] applied a 1 cm sieve to sort out native earthworm cocoons, stating this mesh size was too large to disrupt soil aggregates, replacing the soil into field-installed mesocosms in a rubber plantation, inoculating 30 *P. corethrurus* per mesocosm.

### 3.4. Behavioral extraction

The behavioral response methods are often considered “non-destructive” and indeed they are in comparison to the disruption of soil horizons, texture, and aggregates likely resultant of the application of hand-sorting. Nonetheless, there are inherent biases and non-target effects in behavioral extraction techniques along with other methods discussed here.

#### 3.4.1. Vermifuges

The first set and most common of the behavioral methods involve liquid earthworm irritants applied to soils for extraction at the soil surface. When effective, these liquid expellants can be considered vermifuges.

##### 3.4.1.1. Formalin

First evaluated by Raw [87], the application of dilute formalin to a known area of soil to expel earthworms has since become widely used. Raw [87] found that formalin yielded the highest abundance count for *L. terrestris* (anecic) compared to counting burrow openings at the surface, hand-sorting, and the application of another more lethal irritant potassium permanganate. However, numbers for *Allolobophora chlorotica* (endogeic) and *Eisenia rosea* (epigeic) were much poorer. Reviewing this method, Coleman et al. [78] concluded that formalin is better for vertical burrowing (anecic) species, less for horizontal burrowing species, and ineffective for megascolecid species. In addition, climate restricts efficacy in cold (below 8°C), or very wet or dry soils. Furthermore, as the flow path of formalin cannot be determined, it is difficult if not impossible to determine the volume of soil sampled with this method. However, formalin extraction may be the best technique for *L. terrestris* and similar deep-dwelling species during times of highest activity (spring and fall in temperate regions). No mentions on non-target effects were listed.

Coleman et al. [78] suggest when both shallow and deep dwelling species are present; formalin can be applied to the bottom of a hand-sorted pit and included in total estimations (as done in [26, 86]). Gunn [88] found formalin killed clover ground cover where applied with inhibited recovery, suggesting residual effects on vegetation, thus organic matter and nutrient inputs to the soil system. It is apparent that formalin may have applicability in estimating earthworm populations but may be inappropriate in manipulation studies due to its carcinogenic qualities and possible lasting nontarget affects. Burtelow et al. [57] use formalin not for exclusion but to assess populations after termination of experiment.

### 3.4.1.2. Mustard

A non-toxic alternative to formalin that acts largely in the same way is “mustard flour” or “hot mustard” with the active ingredient allyl isothiocyanate. Gunn [88] concluded that mustard was an effective vermifuge, with better extracting efficiencies than formalin, potassium permanganate, and household detergent. Furthermore, unlike the other extractants tested, mustard had no phytotoxic effect on vegetation nor killed the earthworm specimens given they were rinsed soon after extraction. Chan and Munro [89] compare the effectiveness of mustard to formalin at different concentrations and hand-sorting for anecic and endogeic species in Australia. Through field tests, they found the optimal mustard solution was created by adding 106 g dry mustard powder to 1 L of 5% acetic acid and shaking over-night. This solution is then diluted with water to a 15 mL:1 L ratio. For the anecic *Anisochaetae sp.*, the mustard solution yielded a higher (67%) abundance than formalin. Hand-sorting to 10 cm depth proved to be less efficient than both repellents, as the anecics were able to retreat to lower depths. However, it is important to note that most earthworm studies that employ hand-sorting often sample beyond the 10 cm depth. For the endogeic species *Aporrectodea trapezoides*, all repellent treatments were deemed inefficient, formalin better than mustard with an extraction efficiency of 36%. However, subsequent sorting of repellent treatments revealed that the endogeics were dead. This could reflect that mustard may not be a good extraction technique for endogeics but is appropriate in effective reductions if *in situ* death and decomposition is not a concern. In addition, this study supports previous suggestions that higher concentrations of repellent are too strong for juveniles, preventing their surfacing and thus can cause an underestimation.

Lawrence and Bowers [90] evaluated the use of mustard solution for extracting earthworms by subsequent hand-sorting over a variety of soil and land-use types. They used a solution of 50 g hot mustard powder (Champon™ -0.2% allyl isothiocyanate) mixed with 100 ml water, which sat for 4 hours and then was diluted with 7 L of water. They conclude mustard is an appropriate method as it explained 83% of the variation in total abundance and 98% of total biomass with no differences across land-use types or soil attributes. They do report a decreased efficiency in extracting the endogeic *Octolasion tyrtaeum* compared to other species. This may be due to the rare occurrence of surface openings to burrows of endogeics. It is clear that the extractants discussed so far are biased toward anecics or other species with burrow openings at the surface, with limited efficiency in extracting endogeics. Furthermore, the application of mustard and formalin depends on infiltration qualities of the soil to take effect and may be inappropriate in compact or low-porosity soils such as many clay-rich tropical soils.

Zaborski [91] was the first to take the active ingredient in mustard, Allyl isothiocyanate (AITC) and use it directly as a vermifuge, finding no difference in efficacy in total numbers or biomass compared to formalin. Gutiérrez-López et al. [92] took this expellant one step further, utilizing AITC solution and tested against hand-sorting, formalin, and a combination of methods in a Mediterranean climate in Central-Western Spain with historic Dehesa agroforestry land use. They found that hand-sorting alone had the potential to underestimate anecic species that may escape through burrows out of the sample area or to deeper soil horizons in response to the vibrations of digging researchers, while a combination of hand-sorting and an expellant

minimized this effect. Similar to other studies, they found expellants to be efficient in sampling epigeics, and to an extent, anecic species, but lacking in efficacy in sampling endogeics which most commonly occupy horizontal burrows, making infiltration difficult.

#### 3.4.1.3. *Onion*

Similar to mustard, cultivars of the genus *Allium* (onions) produce natural sulfur compounds in high densities that act as irritants to many animals including humans and earthworms. Steffen et al. [93] tested the application of an onion solution as a vermifuge in both a sandy Ultisol and a clayey Oxisol compared to formalin. The solution was prepared by the authors using white onions blended with water and then strained. Results indicated that 175g onion extract L<sup>-1</sup> was the ideal concentration, with higher concentrations yielded less earthworms, and lower concentrations being less efficient than formalin. This study demonstrated the efficacy of a low cost homemade vermifuge. The authors advise that repeated tests of this expellant are needed in a variety of climate conditions, and that perhaps the compounds themselves could be isolated and utilized.

#### 3.4.2. *Grunting*

Catania [94] reviews the practice of collecting the endemic *Diplocardia mississippiensis* earthworm in Florida's Apalachicola National Forest by locals for generations in a method known as "worm grunting" (or fiddling, snoring, or charming). This involves driving a wooden stake into the ground and then rubbing a metal bar across the top, sending vibrations down the stake and into the ground. Earthworms emerge up to 12 m away and thousands can be collected in hours. The study by Catania [94] supports the hypothesis that worm gruntes unknowingly are mimicking the vibrations of American moles (*Scalopus aquaticus*) the earthworm's natural predator, which they exit their burrows to escape, rejecting an alternative hypothesis of mimicked raindrops. Other predators, wood turtles and herring gulls have been reported to exploit this relationship as well. An interesting question is whether this method is effective in ecosystems where moles or other fossorial predators have never been present (or other animals exploiting this relationship) for earthworms to co-evolve alongside.

#### 3.4.3. *Heat (Kempson apparatus)*

Some researchers have utilized the concept behind the Berlese or Tullgren funnel, used widely to extract diverse groups of soil micro- and mesofauna in the lab, for earthworms, also known as a Kempson apparatus. These devices exploit the photophobic reaction of soil fauna to temperature, light, and moisture gradients, which move away from the heated surface of a soil sample and into a collection pan holding a euthanizing agent or fixation solution. Tuf and Tvardik [95] describe such a device and how to easily build one at low-cost that uses heat from a light bulb to extract fauna based of previous designs. Several research groups have used a modified Kempson apparatus successfully to assess earthworm communities, or as a parameter for land-use or rehabilitation [61, 96, 97]. However, it is obvious that adapting this technique to field settings is not feasible. Furthermore, even for microcosm and mesocosm studies, the nontarget effects on all other soil biota groups are well known,

and consequences of drying on soil structure and aggregates are profound. Therefore, this method is only advised for when complete and destructive sampling is warranted, and suitable access to a lab is provided.

#### 3.4.4. *Electroshock*

An earthworm extraction method that shows great potential in limiting both physical soil disturbance and nontarget species effects is that of electroshock extraction. Satchell [98] first proposed and tested the application of electric current to soils to extract earthworms in a temperate pasture in cases where both contamination by chemicals or physical disturbance might negate experimental factors in field investigations. His results showed that current greater than 0.5 A is necessary for earthworms to be expelled (even with voltages up to 50,000 V), and direct current to be ineffective compared to alternating current. Satchell [98] used a metal probe driven to 18 in (45.7 cm), which he further developed into an elaborate water-cooled double-tube copper electrode to eliminate over-heating and drying of the soil allowing “indefinite” operation. Concentric bands of aluminum to assess distance effects surrounded the probe. The use of 3 A resulted in death of earthworms near the electrode. He concluded that 50-cycle frequency AC was ideal (deviation from this gave no advantages) for 40 min (longer brought more worms but did not change community composition estimates).

Rushton and Luff [99] further evaluated the application of electrical current to sample earthworms in a temperate grassland. Their setup consisted 15 bars surrounding a central electrode in a circle and driven to 30 cm depth. They concluded that ideal current for extraction is between 0.2 and 0.4 A. Rushton and Luff [99] found that juveniles were extracted more efficiently than adults across species and that extraction efficiency was correlated with soil moisture content but not temperature. The latter may be explained by the behavior of earthworms to aestivate during dry conditions and that soil moisture is necessary for electrolytes and the passage of electric current. They note the difficulty in quantifying this method considering the inability of defining where the current flows and thus volume of soil sampled.

Schmidt [100] describes the use of “Worm-Ex III” based on German Thielemann design of 8 stainless steel electrodes (60 cm length, 0.6 cm diameter) arranged in opposing pairs and installed to a depth of 40 cm. Vegetation was clipped and litter removed in the 0.125 m<sup>2</sup> sample area. An auto battery (12 V, 90 Ah) and control unit was used to regulate output voltage and current between specific pairs of electrodes. Voltage was increased in a stepwise fashion from 200 to 600 V over 35 min. Following application, the top 5 cm of soil was sorted with a hand rake.

Schmidt [100] tested this octet design of electroshocking against formalin and hand-sorting in an agroecosystem in Ireland across conventional and direct till on soil of medium to heavy texture over 2 years. He found electroshocking to yield higher numbers and biomass of earthworms compared to formalin extraction, similar community size, and composition compared to hand-sorting, except where recently ploughed. However, electroshocking appeared to underestimate juvenile endogeic and the very small *Murchieona minuscula* (endogeic) compared to hand-sorting. This may be due to the lack of surface burrow openings and leads the author to suggest a subsequent shallow hand-sort following electroshocking. Schmidt [100]

concludes formalin extraction may be better for large anecics (electroshock yielded relatively lower and more variable results for *L. terrestris*) in this temperate agriculture field. Electrical extraction is limited by soil moisture; however this does not present a problem in many of earth's biomes, including temperate and tropical wet forests. Despite its advantages, Schmidt [100] warns that this method is less straightforward, involving many factors that can be altered by individual investigators in both hardware and application, making it more difficult to standardize and/or compare across studies. For these reasons, we suggest all investigators exploiting this method report the electrical current (in amperes) to act as a common denominator making this technique and results comparable. Measurement of current reflects and is a function of such soil properties as moisture and resistance. The clipping of vegetation and postshock hand-sorting of Schmidt's method may introduce some undesirable disturbances.

Eisenhauer et al. [101] tested the same octet design as Schmidt [100] against mustard extraction in dry conditions in a seminatural grassland in Germany. Electrical current was applied for 35 min per treatment in step-wise incremental increase of voltage from 250 to 600 V with no report of electric current or distance between probes. Neither the mustard nor electroshock method improved by addition of water. Mustard extraction was found to be more efficient in sampling anecic earthworm species, even under dry conditions. Endogeic species were extracted in low numbers for both methods compared to hand-sorting, suggesting decreased activity or inactivity during dry seasons. These findings led Eisenhauer et al. [101] to conclude that the octet method was inappropriate in estimating earthworm community structure, however these conclusions may have limited applicability to dry or seasonally dry soils.

The majority of the literature concerning the octet design is in German and finding this device outside of Europe is difficult. However, Weyers et al. [102] have described in detail how to build an octet device with current less than 1 A, including a control panel with data logger capabilities. The authors describe a difference in their design relative to previous octet construction in that this design lacks a return path that would otherwise limit the field, thus earthworms may surface outside of the sample area (which should not be counted if doing an area estimate). Weyers et al. [102] tested their device on conifer soils in North Carolina and agroecosystem soils in Georgia (USA). They listed water as a limiting factor and that higher numbers were obtained in the spring and fall when moisture was optimum for earthworm activity. Furthermore, they note that compaction or thick root-mats can limit the installation of probes or the exit of earthworms. In addition, they list the appropriate safety precautions. They mention that the octet device did not operate under very high soil moisture conditions as soil conductivity limited the generated electrical field. This may suggest why the octet device has not been applied successfully in the humid or wet tropics.

The octet design appears to be a sufficient method for sampling earthworm populations, but the limited surface area affected makes it inefficient for large field exclusion experiments. Bohlen et al. [103] are the first to describe the application of electroshock for large field manipulations. Enclosures of 20.25 m<sup>2</sup> in an agroecosystem were reinforced with PVC walls. Eight steel probes (50 cm long, 33 cm apart) were applied 220 V (AC) for 45–60 min (current not reported). Application was during the known peak of earthworm activity of spring and fall (twice within 2 weeks for each plot) for 3 years. Results revealed earthworm abundance was

reduced 25–75% of natural levels using this method. Bohlen [86] concludes removal by electroshock is much more effective at manipulating earthworm populations than additions in paired plots, possibly due to mortality from handling or resource limitation. Both Blair et al. [38] and Shuster et al. [40] utilized the same plots and methods of above [86]. Blair et al. [38] found no effect of this electroshock application on enchytraeids, nematodes, springtails, mites, or other microarthropods.

Based on methods of Bohlen [86] Costello and Lamberti [23] are the first to apply the electroshock method to earthworm exclusion treatment plots (0.25 m<sup>2</sup>) in a natural system in their Northern temperate deciduous forest site (mixed coarse-loamy, superactive, nonacid soil). Electrodes were placed to 25 cm depth and supplied with 110 V (AC) for 40 min (20 min, then 90° rotation of probes). Upon termination of their experiment, they conclude that electrical reduction successfully excluded *A. caliginosa* and *L. terrestris*, yet was inefficient in expelling juveniles of *Lumbricus* spp., which promptly reverted to the electroshocked plots. Even though the reduced plots were not entirely void of earthworms, the authors believed the disparity between the two treatments was great enough to make a case that electroshocking was a valid method to illustrate the effect of invasive earthworms on forest and riparian soils.

Liu and Zou [42] are the first to report application of the electroshock method in tropical soils (clayey Oxisol of Zarzal series). A slightly different design than Bohlen [86] was applied using 9 steel rods connected in parallel, driven to 50 cm depth in 0.25 m intervals and supplied 240 V (AC, current not reported) for 1.5 hour every 3 months. Hand-sorting at termination of the experiment (one 25 × 25 × 50 cm deep subsample) allowed reporting of extraction efficiencies of 85% in their pasture site and 87% in the forest.

Rhea-Fournier [104] also applied electrical extraction in a wet subtropical forest with some modifications. Two strands of aluminum stakes connected in series were installed at intervals of 35 cm, and to 50 cm depth. Current was supplied by a 220 V AC gas-powered generator, controlled by a dimmer-switch, and direction alternated every application. In the first month plots were shocked six times, with voltage increased in a step-wise fashion every 10 min during hour-long treatments. For the remaining 13 months of the experiment plots were shocked monthly at maximum voltage. To calculate current passing through the circuit (soil), the voltage across a 1 Ω standardized resistor was measured and converted to amps using application of Ohm's Law. Hand-sorting of a 25 cm<sup>2</sup> subsample pedon of each plot at experiment termination was used to determine mean extraction efficiencies, calculated by dividing the total individuals or biomass extracted by the sum of the final hand-sort estimates and total extractions from each plot. Extraction efficiencies were greatest for the anecic *Estherella* sp. in terms of both abundance and biomass (86 and 97%, respectively) with the epigeic *Amyntas* sp. comparable (82 and 94%). Extraction efficiencies were notably lower for *P. corethrurus* (abundance: 60%, biomass: 83%), giving total earthworm extraction efficiencies of 60% in terms of abundance, and 80% biomass. These findings suggest difficulty in extraction of endogeic species compared to anecics and epigeics, or alternatively that the exotic invasive *P. corethrurus* has inherent physiological resistance to the treatment or life history traits that allow rapid recolonization [104]. No significant relationships were found between voltage or current, and biomass, abundance, and species of extracted earthworms.

Electrical extraction is not limited to field experiments and has been employed in laboratory microcosm experiments. As mentioned earlier, Fonte et al. [11] applied a modified electroshock method to soil cores (20 cm diameter, 30 cm deep) collected from a Mediterranean climate agriculture experimental site remaining in a PVC cylinder. Water was added to each core to reach field capacity. Four stainless-steel probes were administered 2 A of electric current for 8 min total (current switched between opposing probes every 2 min). Earthworms were then added to a subset of cores while the others were shocked every month. Importantly, unlike the majority of reports on the electroshock method, this study reports the current applied. Alike, Willems et al. [14] inserted two thin metal probes along the perimeter of a soil core collected from a temperate agriculture field and a PVC sleeve to apply electric current, however very little detail is included in the description of this method.

Staddon et al. [105] designed an experiment to directly test for nontarget effects in temperate European grasslands. The electroshock setup they used is more akin in size to the soil core experiments. It involves a stainless steel cylinder driven into the earth (40.5 cm diameter, 16 cm deep) with a copper electrode installed in the center to a depth of 30 cm, and electrical current was applied at 120 V AC for 4 min (current not reported). Previous trials indicated no greater numbers of earthworms were obtained with more time or voltage; however, this may have been due to the limited volume of soil affected by electric current. As for nontarget effects, the results of Staddon et al. [105] found no effect of electroshocking on canopy CO<sub>2</sub> exchange, root respiration nor mycorrhizal fungal abundance or vitality.

Szlavec et al. [106] applied the electroshock method in a highly replicated field experiment in a deciduous temperate forest in Maryland, USA with mixed results. Their experiment involved trenched plots with aluminum and copper rods installed to 0.4 m, applied with 110/120 V AC electricity for 45 min per application, eight times each. Earthworm reductions of 50% in terms of abundance and biomass were achieved after 2 years of treatment. The authors discuss how the electroshock method was more successful in past experiments in grasslands and agroecosystems, citing such differences as woody underground biomass and spatial heterogeneity inherent in forest ecosystems as impairments for soil conductivity. They advise monitoring soil temperature and moisture, and that a dynamic schedule for electroshocking is adopted in future applications of this method to maximize efficacy. Additionally they recommend strong considerations given to safety, site access and maintenance, labor/effort, and trade-offs between site disturbance and treatments when designing a study using the electroshock method.

To summarize, the findings by Blair et al. [38] and Staddon et al. [105] suggest that any nontarget effects of this method are limited or undetectable, making it ideal for earthworm exclusion experiments that do not aim to reduce other soil fauna. Electrical extraction is the least destructive and thus more desirable among other methods with no reliance on hazardous materials. Furthermore, this approach appears applicable with mixed success in both temperate and tropical forests. It is ideal for study sites such as reserves, protected areas, or long term research sites where introduction of chemicals or interference with other research is undesired [98]. However, researchers who do not include applied electrical current should be strongly criticized against doing so. The actual electrical current felt by the earthworm in

the soil is a result of the soil resistivity and the voltage applied. Knowing that each soil has a different moisture regime dependent on climate and life zone, reporting only the voltage is not very useful in comparing studies. Furthermore, measuring the actual current in the soil is relatively easy by connecting an in-series standardized resistor and measuring the voltage drop across it with a voltmeter.

### **3.5. Comparisons between methods**

Looking at human impacts and disturbance on native vegetation and soil fauna community in a mixed subtropical wet forest in Brazil, Baretta et al. [107] compared hand-sorting of two different sizes of soil pedons to formalin extraction. They concluded that a combination of hand-sorting of larger soil monoliths and formalin extraction was the only proper technique to sample the surface-active and geophagous species.

In experimental meadow grassland in Austria, Čoja and others [108] test five of the methods discussed above in their ability to extract earthworms. They found that hand-sorting, and a modified Kempson apparatus were the two most effective in terms of earthworm abundance, yielding more than three times as many as an electrical octet method. However, it should be noted that for this method soil samples were removed from the field and processed in a laboratory setting. Comparisons between formalin and the mustard extract Allyl isothiocyanate (AITC) in this study yielded no difference, suggesting the nontoxic AITC be used as an alternative. They found an electrical octet method to be biased to juvenile earthworms, compared to other methods applied at their site, resulting in underestimations of biomass. Despite this, they suggest the octet method for sensitive sites, or where groundwater quality is of concern for chemical methods. They concluded that no one technique fulfilled all criteria of low-cost, nondestructive, efficient, and time-saving.

## **4. Quantifying influences of soil fauna on soil processes and biogeochemical cycling**

Considering the myriad of approaches discussed above to passively monitor earthworms or manipulate their populations in the field or laboratory settings, we will now briefly discuss some approaches to quantifying the potential direct and indirect effects of earthworms on the soil ecosystem.

### **4.1. Physical soil properties**

The influence of earthworm casts and burrows on soil porosity can be quantified using a soil infiltrometer between different earthworm treatments. Differences in infiltration rates can serve as corollaries to soil porosity and aeration, which in turn can be indicative of soil saturation rates and microbial processes. Quantification of the impact of earthworm casts on soil aggregate structure and size classes can be directly obtained by using different size sieves on soil samples from different earthworm treatments or communities [7].

## 4.2. Leaf litter consumption

As discussed earlier, certain functional groups of earthworms can drive the rate of forest leaf litter decomposition and/or incorporation into lower soil horizons. Comparing mass loss rates between different mesh size leaf litterbags or between earthworm treatments may allow deduction of the direct influence of the given earthworm community on these processes [45, 62–67].

## 4.3. Soil moisture and groundwater chemistry

The role of earthworms in leachate loss, groundwater, and soil moisture chemistry is a pertinent investigative question, especially when budgeting biogeochemical processes for a given forested watershed. Soil lysimeters can be employed to collect and measure soluble chemical species in soils [17, 18, 22]. For fully saturated soils simple pan lysimeters can be used to collect groundwater samples using gravitational properties. For unsaturated soils, suction lysimeters provide the means to sample soil moisture otherwise held in soil pores by capillary forces by applying negative pressure. These soil water samples can then be analyzed for concentrations of soluble chemical species that may have implications for soil biota, plants, and stream input/output budgets in forests.

## 4.4. Soil chemistry

In studies investigating changes or differences in soil chemistry (such as carbon or nitrogen content) over time, direct quantification of the elements under study can be determined using an elemental analyzer on soil samples at different time steps or at termination of the study across earthworm treatments or communities. Further contrast between earthworm influenced soils and control samples can be achieved by leaving aggregates intact during throughout processing and comparing to samples with aggregates disrupted before chemical analysis. Assuming earthworm casts plays a dominant role in aggregate formation, this method allows enumeration of the quantity of nutrients or carbon are protected within aggregates, and thus more stable, contributing to longer turnover times in soils [21, 59].

## 4.5. Soil microbiota

Recognizing the role of earthworms in regulating microbial activity and processes, it is often desired to quantify the amount of carbon and nitrogen contained within the microbial biomass. Chloroform-fumigation techniques can be applied in the laboratory setting to lyse microbial cell walls, making nutrients contained within the biomass available for measurement [57].

Microbial activity in soils can be measured *in situ* using soil respiration techniques. The most common class of soil respirometer instruments utilizes an infrared gas analyzer. Soil respiration allows for an easily obtainable proxy for heterotrophic metabolic rates in soils, which can be very useful to quantify differences between earthworm treatments and communities. While soil respiration may include the rates of tree root respiration, this can be avoided by trenching sample plots to the depth of existing roots, or otherwise removing plants of substantial size across plots prior to experiment initiation.

As methods for determining microbial communities and functional groups in soils advance through genetic barcoding and other DNA techniques, potential for determining the direct influence of earthworms and other soil fauna on soil microbial communities continues to grow.

To assess the fungal component of soil microbiota separately, a direct count method to determine biovolume can be employed. Creating a soil slurry with agar allows suspension of fungal hyphae fragments to be placed on a microscope slide and counted across a transect [109].

## 5. Conclusions

This review of various techniques and findings shows that there is no single method that can be applied across ecosystems for equally successful earthworm sampling. We suggest a combination of a behavioral extraction technique (such as electroshock or a nontoxic vermifuge) with limited hand-sorting as a viable method for manipulating populations in field experiments. When selecting an extraction (or exclusion) method to apply such site-specific conditions as earthworm community (or functional groups present), soil conditions, and previous land use must be considered. It must be recognized that complete exclusion is not a likely attainable goal in most circumstances. Furthermore, functional group bias may exist for all methods. Both large differences in size and behavior between earthworm species in a given community introduce greater complexity and thus difficulty in calibrating methods to varying ecosystems. For electrical extraction, continual and frequent application is suggested in heavy clay tropical soils, especially where invasive species exist. Sustained methodological development and standardization of these techniques (e.g., electrical current) are encouraged for its utility, particularly in forest ecosystems.

For the comprehensive study of earthworms' roles in forest soils, we advise a combination of field experiments, and laboratory microcosms or controlled mesocosm studies. Recent studies in the genetic structure of common earthworm species reveal that there are likely many undescribed cryptic species only identified through DNA verification, and thus further collaborative efforts to combine morphological traits, phylogenetics, and DNA-barcoding are needed to resolve a possible underestimation of earthworm biodiversity.

## Acknowledgements

This research was supported by grants DEB 0620910, 1239764 and 1546686 from the National Science Foundation to the Institute for Tropical Ecosystem Studies, Department of Environmental Science, University of Puerto Rico, and to the International Institute of Tropical Forestry USDA Forest Service, as part of the Luquillo Long-Term Ecological Research Program. The U.S. Forest Service (Department of Agriculture) and the University of Puerto Rico gave additional support. Additional support was provided to GG by the Luquillo Critical Zone Observatory (EAR-1331841). We would like to thank William McDowell, Jorge Ortiz, and Xiaoming Zou for their contributions and suggested revisions of early versions of this chapter.

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