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Using earthworms as model organisms in the laboratory: Recommendations for experimental implementations

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ABSTRACT

Earthworms are used in an increasing number of microcosm experiments that investigate their behaviour and biology or that consider earthworms an environmental factor that influences soil properties and biological interactions. However, there exists no standardized protocol for performing comparable studies. After giving a short overview of the different experimental approaches using earthworms as model organisms, the present paper provides recommendations for the planning and execution of earthworm experiments that help in achieving comparable results. The recommendations, summarized in a workflow diagram, pertain to the acquisition, treatment and description of earthworms for experimentation, the description and preparation of test soils and the criteria that should be met for valid experimental results.

Introduction

In soil ecology, microcosms are widely used as experimental units to study functional patterns and processes related to soil organisms under controlled laboratory conditions. Pros and cons as well as the relevance of results to the field situation have previously been explicitly discussed (e.g. Teuben and Verhoef 1992; Verhoef 1996; Kampichler et al. 2001). Earthworms have been used in a multitude of microcosm experiments either as a response variable, as in experiments investigating the reaction of earthworms to their environment (sensitivity to chemical, physical, and biological environmental stresses/impacts), or as a treatment variable, such as in experiments investigating the impact of earthworms on their environment (physical, chemical, and biotic impacts on soil and ecosystem properties). Microcosm experiments with earthworms have been shown to be valuable tools for (a) understanding the biology of earthworms (Butt et al. 2004) and their sensitivity to environmental stress (Capowiez and Bérard 2006), and (b) investigating the potential that earthworms have in shaping chemical soil processes (Schrader 1994; Binet et al. 2006), in physically influencing soil properties (Schrader and Zhang 1997; Jégou et al. 2001; Schrader et al. 2007), in affecting the performance of soil microorganisms and fauna (Tiunov and Scheu 1999; Milcu et al. 2006a), and (c) investigating earthworm effects on the aboveground subsystem, such as plant and herbivore performance (Scheu 2003; Partsch et al. 2006; Eisenhauer and Scheu 2008; Wurst et al. 2008). The number of papers that present results from laboratory studies with earthworms increased markedly from around 30 in the 1960s to more than 500 in the current decade (Fig. 1). However, no standardized protocol exists for conducting comparable studies. Only in the case of pure ecotoxicological testing of pollutants on earthworms was a framework developed (Greig-Smith et al. 1992) and later transferred to ISO guidelines (ISO 11268-1 1993; ISO 11268-2 1998). Thus, the present article aims to provide recommendations for general implementation in earthworm experiments to assist in obtaining comparable results. Furthermore, the present paper focuses on the relevance of experimental standardization beyond the laboratory situation. Preceding our recommendations is a short overview of different experimental approaches using earthworms as model organisms.

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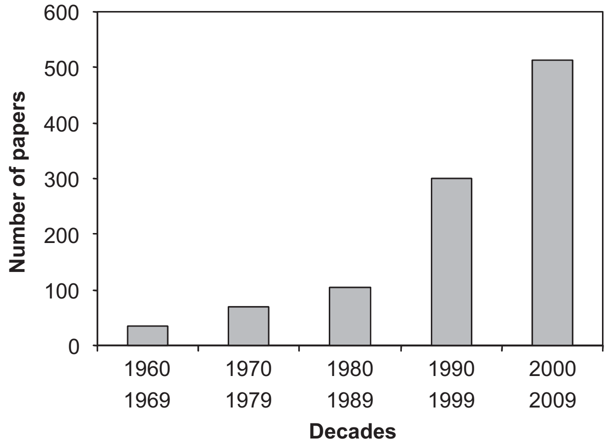

Fig. 1. Number of papers per decade, which present results on laboratory studies with earthworms, obtained from CAB Abstracts (via ISI Web of Knowledge, Thomson Reuters) May 2009; search for topics "earthworm* AND lab*".

Experimental approaches

Biology of earthworms

Aspects of earthworm biology that have been elucidated in microcosm experiments include the following: burrowing and foraging behaviour (Cook and Linden 1996; Langmaack et al. 1999; Bastardie et al. 2003), mating and oviposition (Butt and Nuutinen 1998), mucus excretion and casting of faeces (Scheu 1991; Flegel et al. 1998; Haynes et al. 2003), spatial interaction between earthworms (Capowiez and Belzunces 2001), and plant litter and seed burial (Milcu et al. 2006b; Eisenhauer et al. 2008). In many cases these experiments were performed using soil-filled cuvettes (also referred to as Evans' boxes or two-dimensional (2-D) terraria) (Evans 1947), which easily allow for the visual assessment of earthworm activity. Three-dimensional representations have been achieved with soil-filled PVC cylinders inoculated with earthworms and subsequently analyzed with computerized X-ray tomography (e.g. Langmaack et al. 1999; Capowiez et al. 2006; Schrader et al. 2007). For the observation of earthworm movement in the soil, the "colonne gamma" method with radio-labelled earthworms was pioneered by Capowiez et al. (2001).

Being covered by a secondary water film, earthworms are directly exposed to chemical compounds and metals in soil water. This makes them excellent bio-indicators for toxic soil conditions (Greig-Smith et al. 1992). Much work pertaining to this aspect has occurred in the field of ecotoxicology, resulting in standardization of tests with the species *Eisenia fetida*, which is not a typical soil inhabitant (ISO 11268-1 1993; ISO 11268-2 1998). In some instances soil-inhabiting earthworms have also been investigated in laboratory microcosms (e.g. Mosleh et al. 2003; Lowe and Butt 2005; Capowiez and Bérard 2006). Usually these experiments are performed within simple boxes measuring the survival, reproduction, or other physiological traits of the test animals (e.g. Mosleh et al. 2003). Containers filled with two different substrates allow for testing the avoidance or preference behaviour of earthworms (Topoliantz and Ponge 2003; Capowiez and Bérard 2006; ISO 17512-1 2008).

Earthworm impacts on above- and belowground properties

Due to their feeding and burrowing activity, earthworms enhance the surface of soil organic matter, redistribute the latter in the soil profile, change the biomass and activity of soil microorganisms, and therefore strongly modify soil fertility and nutrient availability to plants (Scheu and Parkinson 1994; Helling and Larink 1998; Haynes et al. 2003). Furthermore, their burrowing activity often leads to a modification of the soil structure, which considerably influences soil water characteristics (Alegre et al. 1996; Shipitalo and Butt 1999; Ernst et al. 2009).

The role of earthworms in nutrient cycling has been investigated in laboratory incubations of earthworms with soil and plant residues in different kinds of containers (e.g. Tiunov et al. 2001; Ernst et al. 2008). In combination with radioactive or stable isotope analysis these experiments helped to reveal specific earthworm-mediated nutrient pathways (Wolters and Joergensen 1992; Jégou et al. 1998; Potthoff et al. 2001; Butenschoen et al. 2007). The effects of earthworms on water infiltration, macroporosity, and soil hydrology have been investigated with 2-D terraria (e.g. Schrader 1993; Bastardie et al. 2005) and with 3-D soil columns (Francis and Fraser 1998; Jégou et al. 2001). Capowiez et al. (2006) used soil-filled PVC cylinders to investigate the effect of earthworms on gas diffusion. The regeneration of compacted soil was studied by Langmaack et al. (2002) in soil columns taken from the field to the laboratory and inoculated with earthworms.

There is now increasing awareness of the importance of earthworms as essential drivers of above- and belowground community and ecosystem processes. Remarkably, impacts of earthworms on plant performance have also been shown to influence the aboveground food web (Wurst and Jones 2003; Eisenhauer and Scheu 2008; Ke and Scheu 2008). Since earthworms are known to be a major component of the decomposer fauna and are amenable to manipulative experiments, they have become a standard tool in ecological research on the importance of soil biota and above-belowground linkages. Research tools include field enclosures and greenhouse experiments. The greenhouse microcosms usually consist of mini-lysimeters made from PVC pipes filled with soil and inoculated with a simple community of earthworms, plants, microbes and sometimes other faunal groups.

Experimental conditions that need to be controlled

Laboratory experiments with earthworms are usually conducted in microcosms. The choice of microcosm type (e.g. column, container, cuvette) and size ought to be determined by the scientific question(s) under investigation, the functional group that the earthworm species belongs to and the timespan of the experiment.

Test organisms (earthworms)

The earthworms used in experiments should be characterized with respect to the following:

- Taxonomic identity (species name): Two reference books are frequently used for the determination of earthworm species: Bouché (1972) in France and parts of Switzerland, Sims and Gerard (1999) in other West European countries. For Austria and surrounding countries there is the key of Christian and Zicsi (1999). However, the different sources are not consistent. Therefore, the identification key should be given together with the species name. Valid species names can be checked against the list of Blakemore (2006). Reference specimens of the earthworm population under study used should be kept (preserved) to allow for the establishment of taxonomic identity.
- Ecological classification: In deciding which earthworm species to use for a laboratory experiment, the behavioural and

adaptational characteristics of the species have to be considered. This can be done in the framework of eco-morphological groups as defined by Bouché (1977; epigeic, endogeic, anecic). From the eco-morphological classification certain constraints for the experimental design can be deduced (e.g. a high risk of earthworms escaping from the experimental container is associated with epigeics, sufficient soil depth for anecics, and adequate food supply for endogeics). Ultimately, however, it is important to keep a species-specific view because each species has its own particular niche (sensu Hutchinson 1957, including food, growth rate, soil preference, etc.). For example, there are great behavioural differences between *Lumbricus terrestris* and *Aporrectodea longa* or *Aporrectodea giardi* (all classified as anecic by Bouché 1977), which may yield quite different effects on soils and ecosystems (Schmidt et al. 2004; Bastardie et al. 2005; Eisenhauer et al. 2008).

- Developmental stage: Five developmental stages are recognized for earthworms: cocoon, hatchling, juvenile, sub-adult (only tubercula pubertatis), and adult (clitellate). Usually, only clitellate and to a lesser extent the sub-adult stages can be determined with certainty to species level using morphological characteristics. On the other hand, juveniles may be more consistent in growth and feeding activity than adult individuals that have more complex behaviours. Usually, however, only clitellate and sub-adult earthworms are used for experimentation.
- Biomass (at the start and at the end of the experiment): Before weighing, earthworms should be put into cold water to remove adherent material from the body surface and briefly swabbed on a tissue. The live weight of an earthworm is strongly influenced by its gut content and its hydration status. Dalby et al. (1996) proposed placing earthworms on water saturated filter paper in order to standardize the tissue water content and eliminate most of the intestinal contents. At 15 °C, gut clearance in Petri dishes with moist tissue can be assumed within 48 h (Eisenhauer et al. 2009). It should be stated explicitly in the experimental methods if worms were weighed with or without gut content. In the field, endogeic earthworms void their guts only when entering quiescence (Edwards and Bohlen 1996). Gut voiding can weaken the earthworms and is not always recommended.
  In experiments where earthworm weight is treated as a response variable, confounding effects of initial weight should be avoided by correcting final weight accordingly. This can easily be achieved by including initial weight as covariate in the analysis of final weight, which then gives a measure of weight change. Similarly, in experiments on earthworm-induced effects on other organisms or ecosystem variables, earthworm biomass may be included as covariate in the analyses of responses of other taxa or ecosystem variables to earthworm activity, especially if the variability of initial earthworm weight is high.
- Physiological status (starvation, dehydration): Earthworms should be in a good physiological condition at the beginning of an experiment (i.e. well-fed and fully hydrated). They reach full hydration when placed in saturated soil for 10–12 h (Stovold et al. 2003) or up to 48 h (Kretzschmar and Bruchou 1991).
- Resting stages: In some species (e.g. genus *Aporrectodea*) earthworms enter diapause and/or quiescence at certain months and/or temperature-humidity conditions (Edwards and Bohlen 1996; Wever et al. 2001). This should be determined in advance when planning the timing and duration of experiments in order to avoid invalid results. Lee (1985) presented an overview on seasonal rhythms and resting behaviour of earthworms and listed many species, which he allocated to the different categories.
- Origin (field collected, laboratory bred, purchased from a commercial supplier): For field-collected earthworms, the collection site, the method used, the time of year and the storage time should be reported. Since the source of commercial supply has also been reported to influence earthworm productivity (Lowe and Butt 2007), this, too, should be recorded.
- Feeding of earthworms: While epigeic and anecic earthworms (detritivores, primary decomposers) can be fed at the soil surface, the food for endogeic earthworms (geophages, secondary decomposers) should be incorporated into the soil (Lowe and Butt 2005). Incorporation of food into the soil is associated with physical disturbance and may only be feasible at the start of an experiment. The monitoring of food consumption by visual inspection is easily done for food on the soil surface but it is impossible for food incorporated into the soil. Therefore, controlled feeding may become a major problem in long-term laboratory experiments conducted with endogeic earthworms. The inclusion of living plants in the microcosm may offer a way to achieve a long-term food supply for endogeics, but in general the food requirements of endogeic earthworms are not yet fully understood and attempts to breed such earthworms continuously in laboratory cultures have only rarely been successful.

In general, care should be taken to ensure that earthworms used in experiments are in good health. Indicators of good health are turgidity, regular body shape without localized constrictions, absence of injuries and epidermal lesions, and mobility. Healthy earthworms (1) react to touch, (2) try to escape from light, and (3) start swimming in water. If earthworms are collected by expulsion (with chemicals or electricity), individuals should be assessed for detrimental effects. Exposure of earthworms to direct sunlight (UV radiation) and high temperature ($>25\,^{\circ}$C) should be avoided and chemical expellants should be washed off immediately with water. Collecting earthworms by formalin extraction should be avoided because plants and soil microflora at the collection site are affected detrimentally (Čoja et al. 2008).

Earthworms should be adapted to the experimental conditions for at least one week before the start of the experiment. This may conflict with a period of gut clearance in moist Petri dishes (if deemed necessary); the adaptation period should be determined with the scientific question in mind. Within a one-week adaptation period a complete exchange of the gut content in the earthworm can be assumed (Pokarzhevskii et al. 2000).

The stocking density should be related to field abundance. Since earthworms are often heterogeneously distributed in natural systems, mean field densities (usually given as number of individuals m^{-2}) should be regarded as a rather rough guide value for laboratory experiments. For most experiments, however, stocking densities should be within the range of observed field abundances (Table 1). In experiments on ecosystem effects of earthworms, the use of different densities covering the range of field densities (10–1000 individuals m^{-2}) might be reasonable to display the range of effects that may occur in natural systems. In data presentation, the experimental stocking density should additionally be transferred to a square metre basis.

Test soil

Soil is a vital component of any experimental system in which endogeic or anecic earthworms are housed. Artificial soils should be avoided, if at all possible, particularly in ecotoxicological

Table 1
Typical ranges of earthworm density and biomass in various biomes (summarized from Lee 1985 and Edwards and Bohlen 1996 in Coleman et al. 2004).

Biome	Earthworm density (ind. m^{-2})	Earthworm biomass (g fresh wt m^{-2})
Temperate hardwood forest	100–200	20–100
Temperate coniferous forest	10–100	30–35
Temperate pastures	300–1000	50–100
Temperate grassland	50–200	10–50
Sclerophyll forest	<10–50	<10–30
Taiga	<10–25	≤10
Tropical rainforest	50–200	<10–50
Arable land	<10–200	<10–50

experiments (van Gestel and Weeks 2004). Within microcosms, soil properties may be compromised or deliberately manipulated compared with those found in the field. To this end, it is vital that test soils are described in some detail with particular respect to

- Soil type and land use.
- Soil horizon.
- Texture.
- Water holding capacity (WHC).
- Water content (at the start and at the end of the experiment). The optimal water content established for soil microbiology (60–70% of WHC) (Parr et al. 1981) may not be transferable to earthworms. Earthworms are very sensitive to soil moisture and some species seem to prefer water contents near field capacity (Wever et al. 2001; Eriksen-Hamel and Whalen 2006; Zorn et al. 2008). Therefore, the adjustment and control of water content is an important part of earthworm experiments. Monitoring of water loss in microcosms can be achieved by weighing. TDR-probes, FD-probes or tensiometers, which can be inserted into soil meso- and microcosms, are also valuable tools for controlling soil moisture characteristics in laboratory experiments.
- pH.
- Organic carbon and nitrogen.
- Bulk density.
- Cation exchange capacity (if possible).
- Pre-treatment of the soil before experimentation.
- Storage time and condition of storage.

It may be appropriate to defaunate the soil before filling the experimental microcosm to avoid undesirable/confounding interactions with other soil fauna. Earthworms and other macrofauna initially present can be removed by hand or sieving (5 mm mesh). In the case of defaunation for earthworm hatchlings and soil mesofauna such as microarthropods and enchytraeids, different methods have been recommended. These include deepfreezing–thawing cycles, the use of microwave radiation, or the application of biocides (Huhta et al. 1989). These methods may also influence soil microbial biomass (e.g. reduction to less than one-third after soil drying at 60 °C for two days; Ernst et al. 2008). This should be considered in studies investigating interactions between earthworms and microorganisms.

We recommend that experiments are conducted as close as possible to natural field conditions (e.g. number of earthworms, organic matter supply, soil conditions). This can be best achieved when there is a field reference for the experiment (Verhoef 1996). Where this is not available, qualified estimates based on the literature should be made. The extent to which earthworms react to the experimental conditions themselves also has to be considered.

Criteria that should be met for the validity of experimental results

- The weight loss of experimental worms (in controls) should be less than 20% in short-term experiments (Bembridge 1998), and less than 30% in long-term experiments. In ecotoxicology, experiments of up to two weeks are considered short term. The effect of the gut content and hydration status on worm weight should be accounted for. A filled gut can comprise about 13% of total earthworm weight (Eisenhauer unpublished: adult *L. terrestris* individuals). Dehydration of earthworms starts at a matric potential of approximately −60 kPa and can lead to weight loss of more than 50% (Kretzschmar and Bruchou 1991; Edwards and Bohlen 1996). There is a need to further investigate the relevance of weight loss in earthworms during experiments.
- The duration of an experiment should be balanced with the nutrition needs of the earthworm species, the soil and food volume in one microcosm, and the number of individuals introduced.
- Experiments with more than 10% of worms (in the control treatment) entering quiescence or diapause should be regarded with caution. Such outcomes might indicate sub-optimal experimental conditions for the given earthworm species.
- In manipulative experiments on the effects of earthworms on ecosystem components, a number of individuals usually disappear from the experimental units, either through escape or due to mortality. Mortality of several individuals might negatively influence health or activity of the remaining ones. In the case of escape, this would lead to an underestimation of the true earthworm effect, making the results a rather conservative estimate. Mortality of earthworms, however, potentially produces a number of undesirable (e.g. fertilization) effects, which may cause misleading results. It is therefore strongly recommended to exclude all replicates from which earthworms disappeared during the course of the experiment from the analyses. Knowledge of disappearance rates in earthworm experiments might be used for planning the necessary number of replicates. Epigeic earthworm species have been reported to have particularly high disappearance rates (up to 80%; Wurst et al. 2008) that might be reduced by installing plastic barriers and/or mesh fences at the top of the experimental microcosm (if of an open nature). At the end of the experiment, care should be taken that all individuals are retrieved from the experimental units, especially in mesocosm studies.
- Field-collected earthworms should be introduced into the experiments preferentially within two to three weeks after collection. Extended storage time compromises the goal of using freshly collected animals.
- Illumination cycles should be performed in consideration of earthworm feeding and burrowing behaviour, to increase the transferability of the results to the field situation.
- Microcosms should be placed randomly in a room/chamber/greenhouse with controlled conditions.

Conclusions

The present paper aims to provide recommendations rather than rules. Many experimental details concerning earthworms will be predetermined by the specific scientific questions addressed. Furthermore, laboratory experiments usually have to

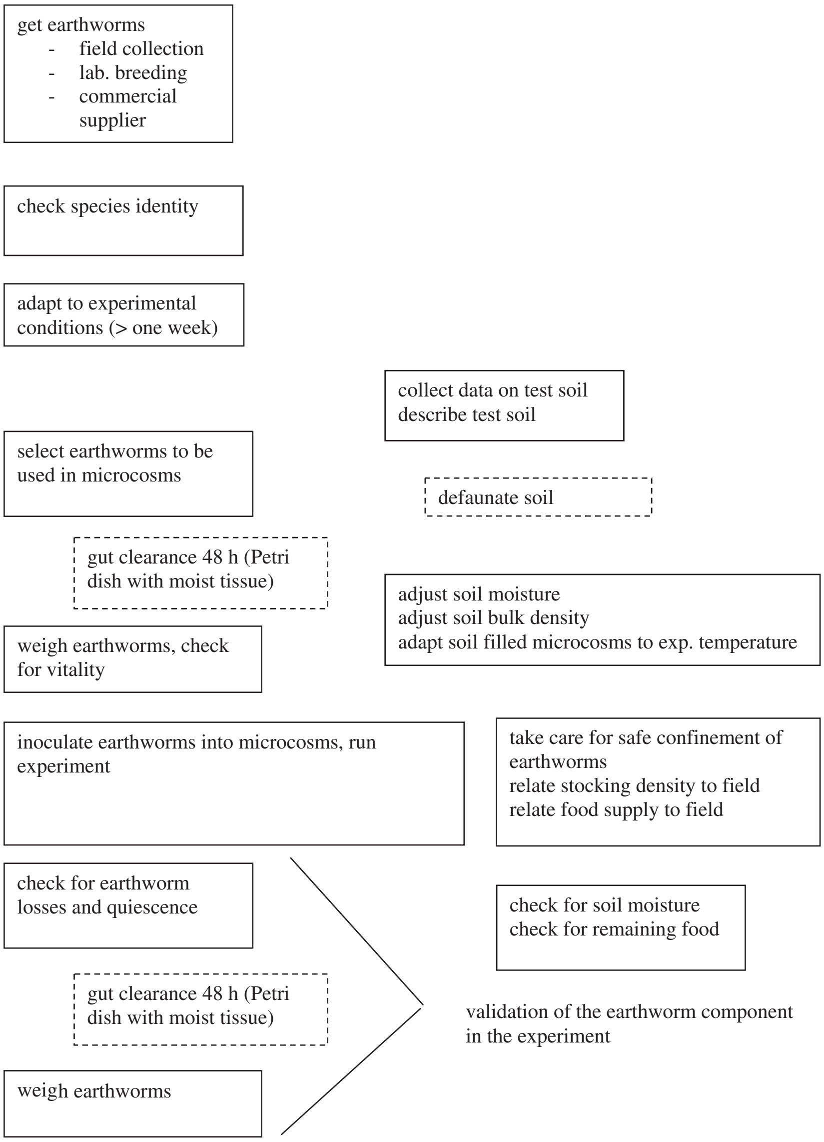

Fig. 2. Proposed general workflow for conducting microcosm experiments with earthworms after all decisions on the experimental setup have been made (boxes with broken outlines for inclusion depending on the specific experimental question).

deal with constraints regarding materials, methods, and the environmental conditions applied. In such cases, we highly recommend a detailed description of all experimental conditions. A number of factors, however, have to be controlled in order to achieve reasonable results. A proposition for a general workflow and the steps to be considered in microcosm (and mesocosm) experiments with earthworms are given in Fig. 2. The importance of the different steps has to be considered in relation to the precise scientific question asked.

As is true for all laboratory experiments, earthworm experiments also focus on single factors or specific impacts and, therefore, fail to re-assemble the natural habitat conditions including all the biotic interactions. With increasing control in laboratory experiments, the variance in data can be reduced but the relation to true field conditions is thereby also decreased. This conflict has to be balanced in every experimental study. Hence, our recommendation to closely approximate natural conditions pertains to the scaling of the experimental setup, rather than trying to mimic nature when experimenting with earthworms.

We hope that these collected experiences will assist researchers who use earthworms as model organisms and will help to avoid known pitfalls. We also anticipate that these recommendations increase the comparability and acceptance of studies from different working groups.

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