Chapter 2
Optimizing Earthworm Sampling in Ecosystems

Jan Valckx, Gerard Govers, Martin Hermy, and Bart Muys

2.1 Introduction

To quantify the role of earthworms in ecosystems, a precise and accurate estimation of their diversity, abundance and biomass is needed. To date, a diverse set of earthworm sampling methods is available, and a diverse amount of chemical expellants have been used to greater or lesser success. Earthworm ecologists have so far often relied on expert judgement or past experience when it comes to spatial sampling designs and determination of sample size. However, based on the ever increasing data available in the literature, we start to understand the spatial organisation of earthworm populations. Moreover, straightforward techniques exist to assess earthworm species richness and the corresponding sampling effort needed to capture it, but so far these were not used in earthworm ecology.

In this chapter, we contribute to the optimization of earthworm sampling in terms of (1) how to sample, (2) where to sample and (3) how many samples to take.

First, we assess optimal concentrations of chemical expellants (allyl isothiocyanate (AITC) and mustard) recently recommended for earthworm sampling. The efficiency of these vermifuges is then evaluated against formalin application using a combined earthworm sampling method (extraction followed by hand sorting). Practical considerations are discussed.

Like many living organisms, earthworm populations are neither uniformly nor randomly distributed, but exhibit an aggregated distribution in patches. The range of spatial autocorrelation in these patches is an important variable to consider in spatial sampling designs. Based on a literature overview, guidelines for spatial sampling design are presented.

J. Valckx, G. Govers, M. Hermy, and B. Muys (✉)
Department of Earth and Environmental Sciences, Division of Geography, Katholieke Universiteit Leuven, Celestijnenlaan 200E, Box 2411, 3001 Leuven, Belgium
e-mail: bart.muys@ees.kuleuven.be

A. Karaca (ed.), *Biology of Earthworms*, Soil Biology 24,
DOI 10.1007/978-3-642-14636-7_2,

Finally, species rarefaction curves are used to determine the optimal sample size to accurately represent earthworm diversity.

2.2 How to Sample? Optimizing Earthworm Sampling Methods

An array of earthworm sampling methods is available, basically belonging to two types: (a) passive (termed physical by Bouché (1969)), where the earthworms are physically sorted from soil, litter and other habitats; and (b) behavioural (termed ethological by Bouché (1969)), where the earthworms are captured after they are moved out from cover (Lee 1985), or a combination of both.

Isolating and hand sorting a soil sample of known volume from the bulk soil has been for a long time the reference sampling method. But this method is labour intensive and time consuming, especially in wet and heavy soils (Bouché and Aliaga 1986; Satchell 1971), and it is impractical in the case of rocky soils, in the presence of a dense root mat or when the study site should remain undisturbed (Bouché and Gardner 1984; Gunn 1992). Anecic species living in permanent vertical burrows (e.g., *Lumbricus terrestris* L.) can reside in or escape to deeper soil layers, making the method inefficient for such species (Bouché and Gardner 1984; Bouché and Aliaga 1986; Chan and Munro 2001). Moreover, cocoons and small juveniles are always underestimated by hand sorting (Bouché and Gardner 1984). Some of these limitations are overcome by washing and sieving the soil samples (Bouché and Beugnot 1972), but this again is a very time (and water) consuming activity.

As an alternative method, earthworms are commonly extracted from their habitat by using chemical expellants. Amongst others, formalin has become the standard vermifuge of the last decades after Raw (1959) demonstrated its superiority over hand sorting. However, extraction efficiency may strongly vary, which may be attributed to deleterious effects of the expellants used, earthworms not being adequately exposed to the chemicals (e.g., due to burrow orientation), or earthworms being unable to react to the irritant substances (e.g., due to low temperature, dormancy) (Daniel et al. 1992). Therefore, efficiency declines from epigeic, to anecic, to endogeic reflecting species behaviour and burrow orientation (Bouché and Gardner 1984).

Despite that its use is recommended in ISO standard ISO/DIS 23644-1 (Rombke et al. 2006), formalin is toxic not only to the earthworms but also to the treated soil system, and its carcinogenic nature poses serious health risks to people working with it (Gunn 1992; Eichinger et al. 2007). Recently, suspensions of prepared mustard (containing variable additives such as vinegar, citric acid, etc.) (Chan and Munro 2001; East and Knight 1998; Gunn 1992) and of dry mustard powder (Chan and Munro 2001; Lawrence and Bowers 2002) were tested as alternative expellants. Chan and Munro (2001) demonstrated that mustard is more efficient than formalin. Gunn (1992) found that it has a comparable efficiency as potassium permanganate and is better than formalin and household detergents. It has consistent

efficiency compared to hand sorting across soil and habitat types (Lawrence and Bowers 2002). Furthermore, mustard does not kill earthworms and shows no phytotoxic effects, as do potassium permanganate and formalin. Unlike formalin, mustard is not a carcinogen (Gunn 1992). However, some disadvantages come along with the use of mustard. Both East and Knight (1998) and Gunn (1992) had difficulties with keeping prepared mustard in suspension at high concentrations, which could influence efficiency. Dry mustard powder suspensions suffer from the drawback that the content of allyl isothiocyanate (further AITC), the active ingredient in mustard seeds believed to irritate the earthworms, is variable and not exactly known. Dry mustard powders are often ground seed mixes of different species (*Brassica nigra* Koch, *Brassica juncea* (L.) Czern. and sometimes *Sinapis alba* L.) and varieties. They all contain precursors of isothiocyanate, and the type and concentration of isothiocyanate produced can vary (Fahey et al. 2001; Zasada and Ferris 2004), also between years and regions of seed production (DeClerq and Daun 2003; Zaborski 2003). Given the known composition and reproducibility of pure AITC solutions and the resulting confidence in the comparability of results, Zaborski (2003) compared pure AITC solutions of different concentrations with formalin and hand sorting and found that 100 mg l^{-1} AITC was as efficient as 200 mg l^{-1} formalin, in particular for the capture of anecic earthworms.

Already in 1969, Bouché acknowledged the complementarities of both passive and behavioural sampling when he suggested his 'etho-physical' method consisting of formalin application followed by soil sampling. The ethological phase of the sampling allows for expelling deep burrowing species, attributing to more accurate biomass estimations, while a soil sample restricted to (e.g.,) 20-cm depth allows for recovery of smaller individuals living near to the surface and of anecics expelled by the ethological method but remaining hidden just below the soil surface, leading to more accurate numbers and biomass. In this chapter, we use Bouché's combined method to compare the efficiency of formalin, mustard and AITC as chemical expellants in earthworm sampling.

We first assess the optimal concentrations of AITC and mustard powder. Working with the optimal concentrations from the first experiment, we assess the earthworm sampling efficiency of formalin, mustard and AITC, using the combined method. Efficiency of methods is compared in terms of earthworm species composition, numbers, biomass, ecological groups (epigeic, anecic, endogeic) and development stages (adults + subadults vs. juveniles).

2.2.1 Material and Methods

2.2.1.1 Preparation of Expellants

Four concentrations (low – medium low – medium high – high) of Indasia™ mustard powder suspensions (0.75, 1.5, 3 and 4.5 g l^{-1}) and AITC solutions (50, 100, 150 and 200 mg l^{-1}) in water were prepared (Table 2.1). Mustard powder

Table 2.1 AITC and mustard concentrations used in the concentration optimization experiment

Expellant	Unit	Concentration			
		Low	Medium-low	Medium-high	High
AITC	mg l^{-1}	50	100	150	200
Mustard	g l^{-1}	0.75	1.5	3	4.5
	mg AITC l^{-1}	45–81[a]	90–162[a]	180–324[a]	270–486[a]

[a]Values from Yu et al. (2003)

Table 2.2 AITC, formalin and mustard concentrations per application in the efficiency assessment

Expellant	Unit	Concentration per application			
		First	Second	Third	Fourth
AITC[a]	mg l^{-1}	75	75	150	150
Formalin[b]	ml l^{-1}	2.5	2.5	5	5
Mustard[a]	g l^{-1}	3	3	6	6

[a]Concentrations based on the concentration optimization experiment
[b]Concentrations from Bouché (1969)

concentrations were based on AITC content in the mustard powder to obtain comparable doses for both expellants (Yu et al. 2003). Mustard powder suspensions were prepared 1 h before application by adding the appropriate amount of powder to 20 l of water and stirring heavily. Just before application, the suspensions were stirred again. Since AITC is not readily soluble in water, pure AITC (95% purity grade, 1.017 g cm^{-3}) was first diluted with isopropanol (technical purity grade, 0.785 g cm^{-3}) to provide a 5 g l^{-1} stock solution (Zaborski 2003). In the field, appropriate volumes of stock solution were then diluted with water to arrive at application volumes of 20 l and the final concentrations mentioned above.

The same procedures of preparing AITC solutions and mustard suspensions were used in the sampling efficiency experiment, using the recommended AITC and mustard powder concentrations from the concentration optimization experiment. Formalin solutions were prepared by diluting the appropriate volume of formalin (technical formaldehyde, ±35%, 1.088 g cm^{-3}) with 20 l of water (Table 2.2).

2.2.1.2 Earthworm Sampling

Expellant concentrations were tested in May 2003 in a regularly mown lawn on a sandy loam soil near Leuven, central Belgium. Earthworms were sampled by each expellant concentration in five replicate plots (0.707 × 0.707 m^2). Expellants were sprinkled with a watering can over the plot area and an adjacent area of 0.1 m outside the plot. Two successive applications of 10 l each with a period of 15 min between applications to collect emerging earthworms were used.

Sampling efficiency of mustard, AITC and formalin was assessed in November 2003 in a harvested wheat field in Court-Saint-Etienne situated in the loam belt of central Belgium. Within a radius of 3 m from eight sampling locations randomly selected in the field, a plot (0.707×0.707 m^2) was randomly located for each expellant. Total expellant volumes of 40 l were applied to the plots with a watering can in four successive applications of 10 l each. During the first two applications, low expellant concentrations were used, which were doubled in the two following applications (Table 2.2), as recommended by Bouché and Aliaga (1986). Between each application there was an interval of 10 min to collect earthworms emerging at the soil surface, corresponding to a total sampling effort of 40 min. Immediately after expulsion, a square soil sample (0.316×0.316 m^2) was dug out to a depth of 0.25 m in the centre of all plots at four randomly selected sampling locations. Soil cores were crumbled and hand sorted during about 1–1.5 h each under favourable light conditions in the laboratory to collect earthworms. All collected earthworms were conserved per plot and per sample fraction (extraction vs. hand sorting) in formalin (5%) immediately after capture. Earthworms were identified following the key in Sims and Gerard (1999) and counted and weighed (with gut content) in the laboratory.

Kruskall Wallis tests (Siegel and Castellan 1988) were used to find differences between methods at a significance level of 0.05.

2.2.2 Salient Observations

2.2.2.1 Concentration Optimization

AITC concentration did not significantly affect the extraction of earthworm numbers or biomass (Fig. 2.1a, b). However, medium-low AITC concentration tended to extract the highest earthworm numbers (Fig. 2.1a), after which numbers began to fall. All but the highest mustard concentrations were less effective in extracting earthworms than AITC, although increasing mustard concentrations consistently yielded higher numbers and biomass (Fig. 2.1a, b).

Five earthworm species were recovered at the lowest AITC concentration (*Lumbricus rubellus* Hoffmeister, *Octolasion tyrtaeum lacteum* Savigny, *Aporrectodea caliginosa* Savigny, *Aporrectodea rosea* Savigny and *Dendrobaena octaedra* Savigny), but only the two largest species were extracted at the highest concentration (*L. rubellus* and *O. tyrtaeum*) (Fig. 2.1c). Increasing AITC concentrations thus resulted in a consistent loss of ability to extract earthworm species in order of increasing average body size. At medium-low concentration *D. octaedra* is no longer recovered (30–40 mm, 123 mg, only one individual recovered), at medium-high concentration *A. rosea* is no longer detected (25–85 mm, 200–216 mg) and at the highest concentration *A. caliginosa* was no longer extracted (40–180 mm, 168–206 mg) (body size ranges from Sims and Gerard (1999)). The total species

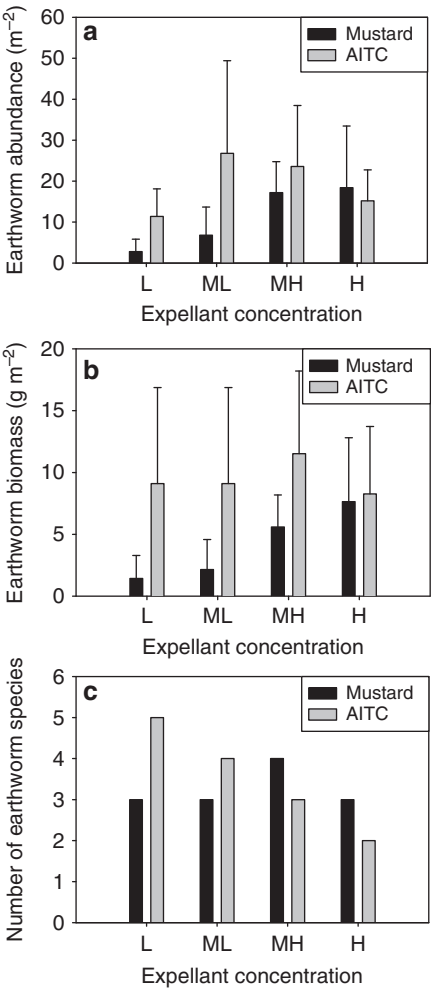

Fig. 2.1 Recovered earthworm numbers ($m^{-2} \pm$ standard error) (**a**) biomass (g $m^{-2} \pm$ standard error) (**b**) and species number (**c**) at low (L), medium-low (ML), medium-high (MH) and high (H) concentrations of mustard and AITC expellants

spectrum extracted by mustard was similar to AITC extraction, but species recovery by mustard was more constant across all concentrations compared to AITC.

2.2.2.2 Efficiency Assessment

Considering chemical extraction only, efficiency was not significantly different between expellants both in terms of numbers (Fig. 2.2a; 121 m^{-2} vs. 109 and 108 m^{-2} for AITC, formalin and mustard, respectively; $p = 0.535$) and biomass (Fig. 2.2b; 73.45 g m^{-2} vs. 70.79 and 58.23 g m^{-2} for mustard, AITC and formalin, respectively; $p = 0.360$). The hand sorted soil samples tended to yield higher earthworm numbers and biomass after mustard extraction than after formalin

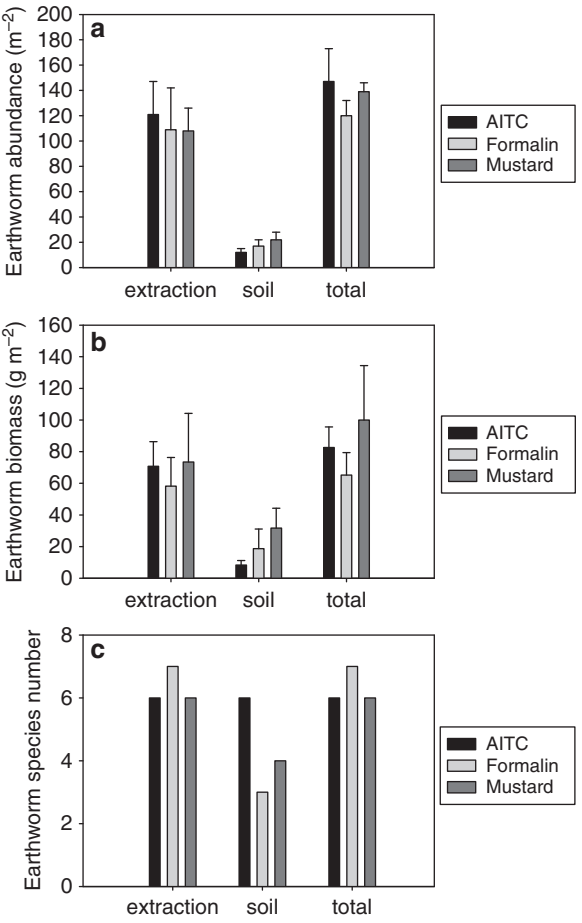

Fig. 2.2 Recovered earthworm numbers ($m^{-2} \pm$ standard error) (**a**) biomass (g $m^{-2} \pm$ standard error) (**b**) and species number (**c**) using different expellants (AITC, formalin, mustard) in a combined extraction – hand sorting sampling method. Results are given per sample fraction and in total

extraction, and contained significantly higher numbers (22 m^{-2} vs. 17 and 12 m^{-2}, $p = 0.037$) and biomass (31.72 g m^{-2} vs. 18.73 g m^{-2} and 8.38 g m^{-2}; $p = 0.015$) than after AITC extraction.

Total sampling efficiency (extraction + hand sorting together) was not significantly different between the three methods in terms of earthworm numbers, biomass and species number (Fig. 2.2). Although not significant, the AITC and mustard method showed slightly higher efficiency in sampling earthworm numbers than formalin (Fig. 2.2a, 147 m^{-2} and 139 vs. 120 m^{-2} for mustard and formalin, respectively; $p = 0.124$). In terms of biomass, mustard sampling tended to be more efficient than AITC or formalin (Fig. 2.2b; 99.99 g m^{-2} vs. 82.68 and 65.17 g m^{-2} for AITC and formalin, respectively; $p = 0.174$).

Six species were commonly recovered by all methods: *Allolobophora chlorotica* Savigny, *A. rosea*, *Aporrectodea longa* Ude, *A. caliginosa*, *L. terrestris* L. and *Octolasion cyaneum* Savigny (Fig. 2.2c). Using the formalin method, also one individual of the epigeic *Lumbricus castaneus* Savigny (0.143 g) was collected. In further calculations, it was omitted. Each expellant was able to extract all six (AITC, mustard) or seven (formalin) of the total number of species found. The soil cores after formalin extraction contained only the endogeic *A. chlorotica*, *A. rosea* and *A. caliginosa*, and the soil cores after mustard extraction additionally contained the anecic *A. longa*.

Although (sub)adults made up only a small portion of the total earthworm numbers (14, 9 and 14% for AITC, formalin and mustard, respectively), they constituted an important part of the earthworm biomass (54, 46 and 54% for AITC, formalin and mustard, respectively). There were no significant differences between the methods (both fractions together) in terms of numbers and biomass of both (sub)adults and juveniles ($p = 0.173$ and $p = 0.593$ for numbers, respectively; $p = 0.219$ and $p = 0.167$ for biomass, respectively). But AITC and mustard marginally extracted more (sub)adult numbers than formalin ($p = 0.060$) and hand sorting after mustard extraction yielded the highest juvenile biomass while the lowest was found after AITC extraction ($p = 0.030$).

Numbers and biomass of anecic species were equally found by the three methods ($p = 0.551$ and $p = 0.309$, respectively). Also comparable numbers of endogeic species (*A. chlorotica*, *A. caliginosa*, *A. rosea* and *O. cyaneum*) were recovered by the three methods ($p = 0.230$), but biomass recovery was marginally higher with mustard than with the other methods ($p = 0.077$). Extraction of anecics did not differ between methods ($p = 0.751$) but the formalin and AITC hand sorting fractions contained more anecic earthworm numbers than mustard ($p = 0.023$). AITC tended to extract more endogeics, both in terms of numbers and biomass, followed by mustard and formalin ($p = 0.076$ and $p = 0.093$, respectively). Hand sorting after mustard extraction recovered more numbers and biomass of endogeics than formalin and AITC ($p = 0.29$ and $p = 0.030$, respectively).

2.2.3 Interpretation

2.2.3.1 Concentration Optimization

In spite of the intentional use of similar AITC concentrations both in mustard and pure AITC treatments, the latter were generally spoken more efficient in sampling earthworms than mustard. Although mustard concentrations were stirred well and sufficiently long during preparation, it is probable that less than the maximum amount of AITC was formed from the glucosinates when mustard was added to the water (Fahey et al. 2001). Furthermore, AITC content of mustard powder is variable. The ranges we found in the literature (Yu et al. 2003) can be somewhat different from the actual content of the powder we used. It is thus possible that the

mustard treatments had an effectively lower AITC concentration than the pure AITC treatments.

The optimum of 150 mg l^{-1} pure AITC for sampling earthworm biomass and the lower optimum of 100 mg l^{-1} for earthworm numbers may indicate a trade-off between the recovery of smaller and more numerous individuals (juveniles and epigeics) on the one hand and heavier and less numerous individuals on the other (adults/subadults and anecics).

The inability to capture smaller earthworm individuals at increasing AITC concentrations could indicate that smaller earthworm species and juveniles cannot tolerate concentrations above 100 mg AITC l^{-1} and therefore do not reach the soil surface. Therefore, following Bouché and Aliaga (1986) in doubling expellant concentrations in subsequent applications, we recommend a concentration of 75 mg l^{-1} AITC in the first two applications, followed by two applications of 150 mg l^{-1} AITC. These concentrations compare very well both with the optimum of 100 mg AITC l^{-1} and the range of acceptable concentrations (60–200 mg l^{-1} AITC) found by Zaborski (2003).

The highest mustard concentrations (3 and 4.5 g l^{-1}) resulted in near-optimum extraction of earthworm numbers, but in a suboptimum recovery of earthworm biomass. This trade-off between numbers and biomass corresponds to the observations with AITC already discussed. Unlike AITC, however, earthworm species and species numbers were stable over all concentrations tested. Chan and Munro (2001) also noted this trade-off effect and found that concentrations between 1.6 and 4.7 g mustard powder per litre were optimal. They further suggested that these concentrations should produce equivalent results to that of English mustard suspension used by Gunn (1992) (15 ml l^{-1}) and better results than formalin (0.55%). They also found that their highest mustard powder concentration tested (~9.4 g l^{-1}) resulted in a significant lower number of collected juveniles compared to the use of lower concentrations. Therefore, we suggest, by analogy to the AITC method and as recommended by Bouché and Aliaga (1986), to use two applications of low (3 g l^{-1}) mustard concentration, and doubling the concentrations (6 g l^{-1}) in the following two applications. A concentration of 3 g l^{-1} lies well within the safe range as found by Chan and Munro (2001) and this study. The doubled concentration of 6 g l^{-1} is suggested by our data for more accurate biomass recovery, and it stays well below the less favourable concentration of 9.4 g l^{-1} identified by Chan and Munro (2001). However, it is advisable to further test a range of higher concentrations than tested here and compare them with standard methods such as hand sorting or with formalin or AITC extraction. From the previous we stress that sufficient mixing of the mustard powder in water is essential.

2.2.3.2 Efficiency Assessment

Overall, the three compared methods (extraction + hand sorting together) did not differ in sampling efficiency. But AITC and mustard extracted earthworms slightly better than formalin. Indeed, AITC and mustard tended to be more successful in

extracting endogeics and (sub)adults than formalin, and also most other tests gave the same trends in extraction efficiency. Interestingly, the greatest differences between methods were found in the soil fraction. On the one hand, the soil samples after formalin extraction contained more numbers of anecics (and not endogeics as could be expected from formalin's lower extraction efficiency) than after mustard extraction (with intermediate values for AITC). On the other hand, more endogeics and juveniles were collected in soil samples after mustard extraction than after AITC extraction (with intermediate values for formalin). If we assume that earthworms found in the soil fraction did not react to the vermifuges, we have to conclude that mustard fails in catching some endogeics and to a lesser extent some juveniles compared to the other expellants. We also would expect inverse results for the chemical extraction and hand sorting fractions but these were not observed. From this complex overall picture, it is not evident to push one of the methods forward as the best.

Characteristics of the expellants other than extraction efficiency may then play a decisive role in choosing between one and the other. Formalin proved to be lethal to earthworms (and other soil organisms and plants) (Gunn 1992; Eichinger et al. 2007), which is problematic if living worms have to be collected. Formalin is also known to be carcinogenic to humans, and in some countries its use is forbidden due to national health and safety regulations (Schmidt 2001). Together with its suggested lower extraction efficiency compared to AITC and mustard, these qualities make formalin a less preferred expellant.

Also some drawbacks with the use of AITC must be noted. First, AITC should be used with great care in the field since it is very toxic to aquatic organisms, and may cause long-term adverse effects in the aquatic environment. Second, the preparation and practical use of AITC solutions is hindered by the toxic and irritating damps of AITC, more disturbing than the ones of formalin. As a consequence, hand sorting after pure AITC application is not recommended for health and safety reasons, and it is not possible without proper protection. Third, earthworms emerging at the surface after AITC application are markedly groggy, even the bigger individuals. This could mean that some individuals, especially smaller ones, can get so drowsy that they do not even reach the surface while fleeing for the irritating AITC. Therefore, it is important to combine AITC extraction with succeeding hand sorting of a soil core to collect the left behind earthworms, which conflicts with our earlier discussion of the irritant nature of AITC. Another important demerit is that in many cases earthworms collected by AITC emerge with a loosened epidermis. This negative effect is most probably caused by the isopropanol in which AITC is diluted and seriously hampers identification after collection.

Mustard powder is a harmless and cheap product with comparable sampling efficiency as AITC. A disadvantage is that the concentration of AITC and its variability in mustard powder suspensions is not exactly known, although the extraction result was quite robust over a range of concentrations (Fig. 2.1). For reliable results, we, nevertheless, suggest to use mustard powder from the same manufacturer and from the same lot if possible and to order quantities sufficient for the whole sampling campaign (Gunn 1992).

2.3 Where to Sample? Optimizing Spatial Sampling Design

Like many living organisms, earthworm populations are spatially neither uniformly nor randomly distributed, but usually occur in spatial clusters. Both spatial heterogeneity and autocorrelation should be accounted for in the sampling design of any ecological field study, as many statistical tests rely on the assumption of independence of observations.

2.3.1 Spatial Autocorrelation and Sampling Design

Observed values of a variable (e.g., earthworm density) are said to be spatially autocorrelated when pairs of observations with a certain distance apart are more similar (positive autocorrelation) or less similar (negative autocorrelation) than expected for randomly associated pairs of observations. This higher (or lower) similarity among mutually closer observation sites is very common in nature. Autocorrelation in fact refers to the lack of independence among the error components of observations due to geographic proximity (Legendre and Legendre 1998). Because of this, the use of autocorrelated data in tests of statistical significance that rely on independent observations (e.g., *t*-test, correlation analysis, analysis of variance, linear regression; clustering and ordination methods do not use tests of statistical significance and may thus be safely used in the case of autocorrelated data) is problematic.

Unless the aim of the study is to analyze the spatial structure in the data per se (by correlograms, variograms, see Rossi et al. 1995 for a comprehensive introduction to geostatistical analysis of ecological data), spatial dependency among observations may be removed to validly use classical statistical tests. However, this is not recommended as discarding observations, until spatial independency is achieved, results in (costly) information loss (Fortin and Dale 2009). Similarly, the use of de-trended data cannot be advised if autocorrelation is inherent to the process under study.

Alternatively, various corrected tests that rely on modified estimates of the variance and on corrected estimates of the effective sample size and the number of degrees of freedom have been developed and their use should be advocated (e.g., Dutilleul 1993). When corrected statistical methods are not available, permutational tests may be used, given that the appropriate procedure of random permutation of observations to determine significance can be specified.

A more proactive way of preventing dependency among observations lies in the careful design of spatial sampling schemes, and will be discussed here. Classical statistical text books tell that without prior knowledge of the phenomenon under study, ecologists should rely on random or systematic sampling designs to avoid dependency among observations. But the random or systematic spatial allocation of observation sites per se does not rule out that observations may be spatially dependent to some degree: this will be the case if the average distance between observation sites is smaller than the zone of spatial influence of the underlying

ecological process. The zone of spatial influence is commonly determined by the range of the variogram model describing the spatial autocorrelation in the data (Rossi et al. 1995).

In recent years, studies addressing the spatial distribution of earthworm populations have yielded insights in the scales over which earthworm populations are spatially related (Table 2.3). Across a range of ecosystems, earthworm species are spatially distributed in clusters ranging from as small as 7 m in diameter (Rossi 2003b) to more than 100 m (Hernández et al. 2007). However, the majority of spatial ranges of autocorrelation lies within 20–50 m. Adult individuals of *L. terrestris* consistently live in small clusters (~20 m) compared to other species, presumably related to its surface mating behaviour. Another general trend is that juveniles tend to inhabit larger patches than conspecific adults.

Samples should ideally be taken at distances further apart than the variogram ranges as given in Table 2.3 for the given ecosystems and species. In practice, this means sampling locations should be at least 20–50 m apart to collect nonautocorrelated independent samples for most species and circumstances. Ideally, the site-specific spatial structure of the earthworm populations under study should be assessed beforehand in a pilot study or this information should be retrieved from previous surveys (Legendre et al. 2002). A risk that arises when spacing the sampling locations, according to the spatial structure, is that not enough independent samples can be collected from (strata within) the observation site or the experimental unit. As always, a good balance between theory and practice must be found, and other techniques (see earlier) can assist in the analysis of spatially dependent data.

2.3.2 Sample Unit Size and Shape

Observed spatial (and temporal) variability is a function of the 'window size' that one uses to look at the world (Levin 1992): as window size (i.e., support size (the volume (sample unit surface area $\times$ depth) collected per sample unit)) is increased, variability will decay. The inversely proportional relationship between window size and variability depends on how spatial autocorrelation decreases with distance within the range of spatial autocorrelation. Additionally, larger study objects need larger support sizes to adequately capture variability. In practice, different support sizes apply to nematodes, mesofauna and earthworms (Stein and Ettema 2003).

Clearly, lower sampling variance by well-chosen sample unit size and shape, in combination with well-chosen (nonautocorrelated) sample unit locations (see Sect. 2.3.1) lead to smaller confidence intervals, and thus more rapid detection of significant treatment differences for similar sampling efforts.

Little is known about the ideal support size in earthworm sampling. For example, many researchers adhere to a square sample unit area of 0.25×0.25 m^2 of variable depth, depending on the species of interest, life stage and season without further

Table 2.3 Overview of the types of fitted models and ranges of spatial autocorrelation from studies describing the spatial distribution of earthworms in a range of ecosystems

Country	Land use	Earthworm taxon	Life stage	Variogram parameters						Reference
				Model	Range	Model	Range	Model	Range	
		Individuals (m^{-2})								
Germany	Arable land	*L. terrestris*	–	Spherical	36					Poier and Richter 1992
			Adult	Spherical	21					
			Juvenile	Spherical	37					
		A. caliginosa	–	Spherical	53					
			Adult	Spherical	28					
			Juvenile	Spherical	53					
		A. rosea	–	Spherical	70					
			Adult	Spherical	50					
			Juvenile	Spherical	–					
Belgium	Arable land	*L. terrestris*	–	Spherical	30					Valckx et al. 2009
			Adult	Spherical	14					
			Juvenile	Spherical	34					
		A. longa	–	Spherical	64					
			Adult	Spherical	40					
			Juvenile	Spherical	63					
		A. caliginosa	–	Spherical	29					
			Adult	Spherical	33					
			Juvenile	Spherical	30					
		A. rosea	–	Spherical	45					
			Adult	Spherical	40					
			Juvenile	Spherical	44					
Canada	Arable land	Lumbricidae		Linear	16					Whalen and Costa 2003
	Hay field	Lumbricidae		Linear	18					
	Deciduous forest	Lumbricidae		Linear	21					
Spain	Pasture			2001		2002		2003		Hernández et al. 2007
		A. rosea		Gaussian	134	Spherical	18	Spherical	26	
		H. elisae		Exponential	41	Spherical	50	Gaussian	103	
		A. caliginosa		sph Erical	60	Spherical	30	Spherical	26	
Ivory coast	Savanna	Eudrilidae		Spherical	27					Rossi 2003a
		Millsonia anomala		Spherical	26					
				1995		1996				Rossi 2003b
		Filiform Eudrilidae		Spherical	8	Spherical	8			

(*continued*)

Table 2.3 (continued)

Country	Land use	Earthworm taxon	Life stage	Variogram parameters						Reference
				Model	Range	Model	Range	Model	Range	
		Individuals (m^{-2})								
				–	–	Spherical	17			
		Hyperiodrilus africanus		Spherical	7	–	–			
Colombia	Savanna			1993		1994		1995		Jiménez et al. 2001
		Andiodrilus sp.	Adult		–		–	Spherical	45	
			Juvenile		–		–		–	
		Aymara sp.	Adult		–		–	Spherical	41	
			Juvenile		–		–	Linear		
		Glossodrilus sp.	Adult	Spherical	37		–		–	
			Juvenile	Spherical	30	Spherical	36		–	
		Ocnerodrilidae sp.	Adult		–	Spherical	31		–	
			Juvenile		–		–		–	
	Pasture	*Andiodrilus* sp.	Adult		–	Spherical	29	Spherical	55	
			Juvenile		–	Spherical	27	Spherical	30	
		Aymara sp.	Adult		–		–		–	
			Juvenile		–		–		–	
		Glossodrilus sp.	Adult	Linear	–		–		–	
			Juvenile		–	Spherical	57	Spherical	42	
		Ocnerodrilidae sp.	Adult		–		–	Spherical	31	
			Juvenile		–		–		–	
		Biomass (m^{-2})								
Germany	Arable land	*L. terrestris*	–	Spherical	22					Poier and Richter 1992
			Adult	Spherical	21					
			Juvenile	Spherical	56					
		A. caliginosa	–	Spherical	35					
			Adult	Spherical	27					
			Juvenile	Spherical	47					
		A. rosea	–	Spherical	38					
			Adult	Spherical	53					
			Juvenile	Spherical	–					
France	Grassland	*L. terrestris*	Adult	Spherical	28					Cannavacciuolo et al. 1998
			Juvenile	Gaussian	18					
		A. caliginosa	Adult	Spherical	30					
			Juvenile	Linear	–					

justification. One of the few studies addressing sample unit size and shape in earthworm sampling (Dickey and Kladivko 1989) shows that the most efficient, acceptable sample unit size–shape was 10 cm along the row (the shortest increment in the experiment) by 45 cm across the row in a *Zea mays* row crop (75 cm inter-row distance) for an earthworm community composed of *Aporrectodea tuberculata* and *L. rubellus*. They also found as much as a threefold difference in the sampling efficiency (by hand sorting) within the range of tested sample unit size–shape combinations. The least efficient size–shape was also the largest (30 cm along the row by 75 cm across the row). The authors state that rectangular quadrants have a substantial advantage over square ones of equivalent sample unit area. Especially for patchily distributed populations, an elongated sample unit shape with its longest axis perpendicular to the expected or known density gradient results in a decreased variance (i.e., across corn rows in this case). The relative importance of sample unit size and shape for other ecosystems, sites, seasons, species and life stages may well differ from the study by Dickey and Kladivko (1989) but hitherto knowledge is lacking. Rossi and Nuutinen (2004), in their study of the spatial distribution of *L. terrestris* L. midden density in a Finnish forest, also found an considerable increase in total variance with decreasing sample unit, while neither the mean midden density nor the global distribution pattern were markedly affected by sample unit size (0.125, 0.25 or 1 m^2). Given that earthworm sampling is a time and labour consuming activity, in practice an acceptable level of sampling variance must be decided upon so that a corresponding compromise between the sample unit size and the number of samples can be found.

2.4 How Many Samples? Optimizing Sample Size

Commonly, earthworm communities are species-poor in a given area or ecosystem; in arable land they usually comprise only 4–6 species (Edwards and Bohlen 1996). Lavelle (1983) relates this to the simultaneous occurrence of species from different ecological categories, the plasticity of earthworm species, and temporal and spatial (both horizontal and vertical) niche separation. Nevertheless, to compare the effects of land use, land management and environmental factors on earthworm communities, it is important that a sufficient number of samples are taken in order to be a 'true' representation of local earthworm diversity.

Therefore, sample-based species rarefaction curves with 95% confidence intervals were computed by the free software Estimates (Colwell 2009), using the analytical formulas of Colwell et al. (2004). Sample-based rarefaction curves plot the expected number of species in a small collection of n samples drawn at random from the large pool of N samples, against a measure of cumulative sampling effort (e.g., the cumulative number of samples) (Gotelli and Colwell 2001). Thus, rarefaction curves represent the statistical expectation of the corresponding species accumulation curves, which themselves record the total number of species revealed as additional sample units are added in one particular random order to the pool of all previously

collected samples (Gotelli and Colwell 2001). Typically, both types of curves rise relatively rapidly at first, then much more slowly as increasingly rare taxa are added. In principle, an asymptote will eventually be reached for samples coming from a homogeneous study area (representing within-habitat α-diversity) (Gotelli and Colwell 2001). The point where the curve levels off is usually accepted as the optimal balance between sampling effort and an accurate estimation of local species diversity.

Species abundance data (number of individuals m^{-2}) from a study of the spatial distribution of earthworm density in an arable field in central Belgium (Valckx et al. 2009) served as input data. Data from two sampling methods (earthworms extracted by mustard powder (0.5 m^2) vs. earthworms hand sorted from a soil monolith (0.1 m^2 to a depth of 20 cm) after mustard extraction) from 30 sampling locations were analyzed both separately and combined to determine which method was more efficient in capturing earthworm diversity. The expected number of species was calculated against both the cumulative number of samples and the cumulative sampled area (m^2). The latter was done to account for differences in sample plot area used in both sampling methods.

Here, we do not discuss the determination of sample size to protect against both type I and type II errors, or to estimate the variables of interest with sufficient precision to detect (ecologically) meaningful differences between treatments. For this, we refer to classic statistical textbooks (e.g., Neter et al. 1996) where the relevant techniques (e.g., the power approach) are explained in much detail.

Figure 2.3 shows that all species rarefaction curves reached an asymptote well before total accumulated sampling effort, whether expressed as number of samples or sampled area. This suggests that species inventory was complete in the study area, that is, 'true' species richness was observed and not a single species remained undetected or rare species were present. Indeed, the occurrence of rare species (unique and duplicates, i.e., species occurring in exactly one or two samples, respectively) may severely hamper to reach asymptotic species richness (Mao and Colwell 2005).

Circa ten samples of 0.5 m^2 extracted by mustard were needed to capture the total observed species richness of six species (*L. terrestris* L., *A. longa* UDE, *A. caliginosa* SAVIGNY, *A. chlorotica* SAVIGNY, *A. rosea* SAVIGNY and *O. cyaneum* SAVIGNY) (Fig. 2.3a). For the same purpose, more than twice as much hand-sorted soil samples ($\pm$25) were needed (Fig. 2.3c). However, rescaling the number of samples to the actually sampled area (m^2) in the *x*-axis revealed that on a per m^2 basis, hand sorting of soil samples is more efficient than mustard extraction to observe the asymptotic local diversity (Fig. 2.3d vs. b): only a total area of $\pm$2.4 m^2 need to be sampled by hand sorting, while a total sampling area of $\pm$5 m^2 is needed for earthworm extraction. This result reflects the lower efficiency of the extraction sampling method compared to the hand sorting method to catch endogeic species richness of the earthworm assemblage dominated by endogeic species in this particular arable land context (4 out of 6 observed species were endogeic). Earthworm communities dominated by epigeic and/or anecic species may well be more efficiently sampled on a per m^2 basis by the mustard extraction method. Using the combined sampling data (extraction + hand sorting) (Fig. 2.3e, f), it is clear that depending on the species composition and how sampling effort is expressed, the

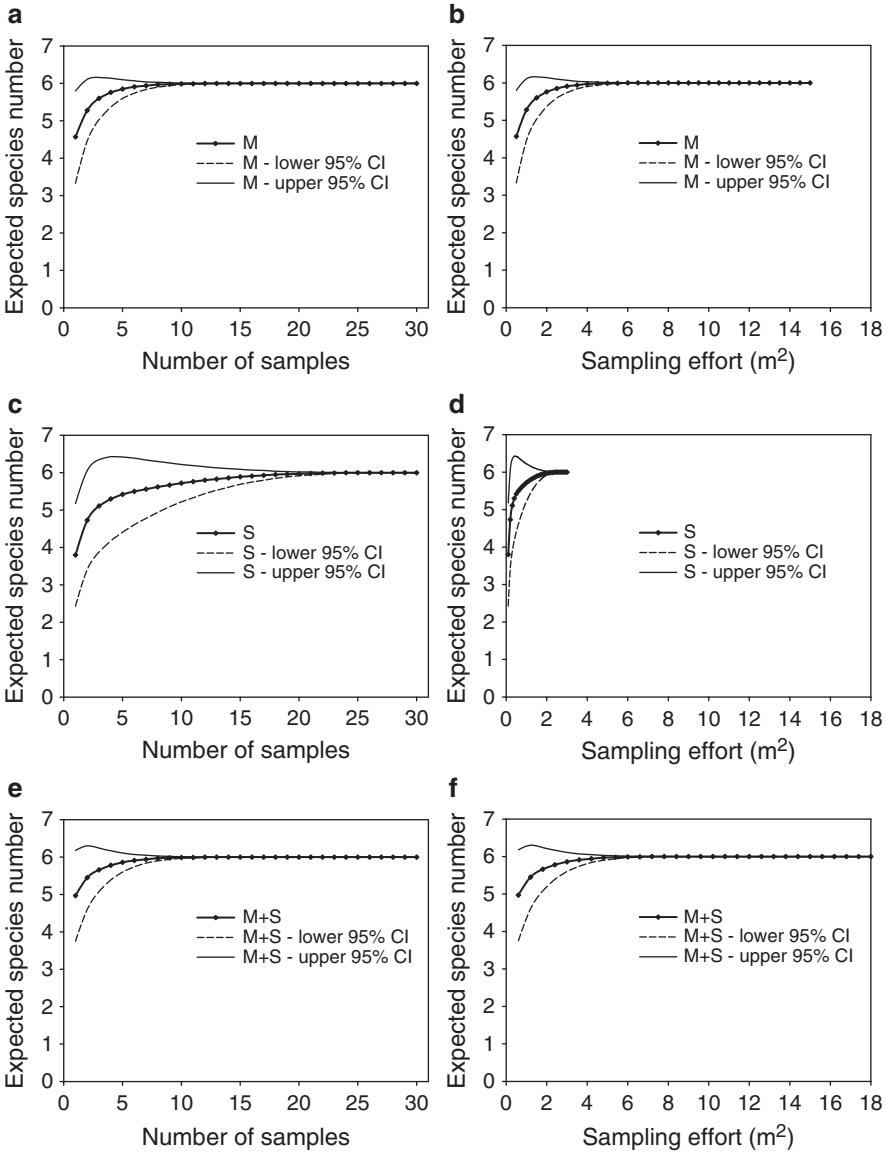

Fig. 2.3 Sample-based species rarefaction curves (*thick lines*) with 95% confidence intervals (*thin lines*) of earthworms collected by mustard extraction (M; 0.5 m^2 plot) (**a** and **b**), by hand sorting a soil monolith after mustard extraction (S; 0.1 m^2 plot) (**c** and **d**), and by a combination of both methods (M + S) (**e** and **f**). *Graphs* on the *left* show the expected species number as a function of sample size; in the *graphs* on the *right* sample size is rescaled to sampling effort expressed as total area sampled (m^2)

combined sampling method may be more or less efficient than its constituent sampling methods.

2.5 Conclusion

Because of the toxicity to the soil ecosystem and the irritant nature to the operators of formalin and AITC, these expellants are less recommended for earthworm sampling compared to mustard. Mustard extraction consisting of two initial applications of low expellant concentration (3 g mustard l^{-1}) followed by two applications of doubled concentrations (6 g l^{-1}) is at least equally efficient as the other vermifuges, and has the advantage to be harmless to the environment.

Sample unit locations should ideally be randomly selected at distances larger than the range of spatial autocorrelation, and sample unit size and shape should reflect the variability to be expected. Much of this important information to assure the collection of independent data is lacking when research projects start, hence the importance of pilot studies. More research is needed about ideal support size.

Species rarefaction curves are a useful and promising technique to assess asymptotic earthworm species richness and the corresponding sampling effort needed to capture it. Despite its wide use in ecology, hitherto it was not applied in the context of earthworm research. Earthworm communities are usually species-poor, which has the advantage that a relatively small number of samples usually suffice to capture total species richness compared to other taxa. However, species rareness may complicate conclusions about the ideal sampling effort because it prevents rarefaction curves to reach an asymptote. It is recommended to explore this technique's potential with data from other ecosystems, with different species compositions and rareness. Also the effects of spatial patchiness on estimation of asymptotic species richness needs further research as it can be assumed that higher spatial aggregation means that a higher sampling effort is needed to capture total earthworm richness compared to randomly distributed organisms.

Acknowledgements We thank Veerle Cielen, Eric Van Beek and Johan Wauters for their technical assistance during field work and earthworm identification.
This research is part of the project 'Framework for assessing sustainability levels in Belgian agricultural systems-SAFE' (CP 04) supported by the Belgian Science Policy Office. Financial support by the IWT project ECOWORM (project 040681) is gratefully acknowledged.

References

Bouché MB (1969) Comparaison critique de méthodes d'évaluation des populations de Lombricidés. Pedobiologia 9:26–34

Bouché MB, Aliaga R (1986) Contre une degradation physique et chimique des sols et pour leur optimisation économique, l'échantillonage des Lombriciens: une urgente nécessité. La défense des Végétaux 242:30–36

Bouché MB, Beugnot M (1972) Contribution à l'approche méthodologique de l'étude des biocénoses. II. L'extraction des macro-éléments du sol par lavage-tamisage. Annales de Zoologie-Ecologie Animale 4:537–544

Bouché MB, Gardner RH (1984) Earthworm functions. VIII. – Population estimation techniques. Revue d'écologie et de biologie du sol 21:37–63

Cannavacciuolo M, Bellido A, Cluzeau D, Gascuel C, Trehen P (1998) A geostatistical approach to the study of earthworm distribution in grassland. Appl Soil Ecol 9:345–349

Chan K-Y, Munro K (2001) Evaluating mustard extracts for earthworm sampling. Pedobiologia 45:272–278

Colwell RK (2009). EstimateS: statistical estimation of species richness and shared species from samples. Version 8.2. User's guide and application published at: http://purl.oclc.org/estimates

Colwell RK, Mao CX, Chang J (2004) Interpolating, extrapolating, and comparing incidence-based species accumulation curves. Ecology 85:2717–2727

Daniel O, Jager P, Cuendet G, Bieri M (1992) Sampling of *Lumbricus terrestris* (Oligochaeta, Lumbricidae). Pedobiologia 36:213–220

DeClerq DR, Daun JK (2003). Quality of western Canadian mustard 2003. http://www.grainscanada.gc.ca/Quality/Mustard/2003/mustard-2003-e.pdf. Accessed July 19 2005. Canadian Grain Commission, Winnipeg, Man

Dickey JB, Kladivko EJ (1989) Sample unit sizes and shapes for quantitative sampling of earthworm populations in crop lands. Soil Biol Biochem 21:105–111

Dutilleul P (1993) Modifying the t test for assessing the correlation between two spatial processes. Biometrics 49:305–314

East D, Knight D (1998) Sampling soil earthworm populations using household detergent and mustard. J Biol Educ 32:201–206

Edwards CA, Bohlen PJ (1996) Biology and ecology of earthworms. Kluwer Academic Publishers, Dordrecht

Eichinger E, Bruckner A, Stemmer M (2007) Earthworm expulsion by formalin has severe and lasting side effects on soil biota and plants. Ecotoxicol Environ Saf 67:260–266

Fahey JW, Zalcmann AT, Talalay P (2001) The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. Phytochemistry 56:5–51

Fortin MJ, Dale MRT (2009) Spatial autocorrelation in ecological studies: a legacy of solutions and myths. Geogr Anal 41:392–397

Gotelli NJ, Colwell RK (2001) Quantifying biodiversity: procedures and pitfalls in the measurement and comparison of species richness. Ecol Lett 4:379–391

Gunn A (1992) The use of mustard to estimate earthworm populations. Pedobiologia 36:65–67

Hernández P, Fernandez R, Novo M, Trigo D, Diaz Cosin DJ (2007) Geostatistical and multivariate analysis of the horizontal distribution of an earthworm community in El Molar (Madrid, Spain). Pedobiologia 51:13–21

Jiménez JJ, Rossi J-P, Lavelle P (2001) Spatial distribution of earthworms in acid-soil savannes of the eastern plains of Colombia. Appl Soil Ecol 17:267–278

Lavelle P (1983) The structure of earthworm communities. In: Satchell JE (ed) Earthworm ecology – from Darwin to vermiculture. Chapman and Hall, London, pp 449–466

Lawrence AP, Bowers MA (2002) A test of the 'hot' mustard extraction method of sampling earthworms. Soil Biol Biochem 34:549–552

Lee KE (ed) (1985) Earthworms, their ecology and relationships with soils and land use. Academic, New York

Legendre P, Legendre L (eds) (1998) Numerical ecology. Elsevier Science B.V, Amsterdam

Legendre P, Dale MRT, Fortin M-J, Gurevitch J, Hohn M, Myers D (2002) The consequences of spatial structure for the design and analysis of ecological field surveys. Ecography 25:601–615

Levin SA (1992) The problem of pattern and scale in ecology: The Robert H. MacArthur Award Lecture. Ecology 73:1943–1967

Mao CX, Colwell RK (2005) Estimation of species richness: mixture models, the role of rare species, and inferential challenges. Ecology 86:1143–1153

Neter J, Kutner MH, Nachtsheim CJ, William W (1996) Applied linear statistical models. WCB/McGraw-Hill, Boston

Poier KR, Richter J (1992) Spatial distribution of earthworms and soil properties in an arable loess soil. Soil Biol Biochem 24:1601–1608

Raw F (1959) Estimating earthworm populations by using formalin. Nature 184:1661–1662

Rombke J, Sousa J-P, Schouten T, Riepert F (2006) Monitoring of soil organisms: a set of standardized field methods proposed by ISO. Eur J Soil Biol 42:61–64

Rossi J-P (2003a) Clusters in earthworm spatial distribution. Pedobiologia 47:490–496

Rossi J-P (2003b) Short-range structures in earthworm spatial distribution. Pedobiologia 47:582–587

Rossi J-P, Nuutinen V (2004) The effect of sampling unit size on the perception of the spatial pattern of earthworm (*Lumbricus terrestris* L.) middens. Appl Soil Ecol 27:189–196

Rossi J-P, Lavelle P, Ebagnerin Tondoh J (1995) Statistical tool for soil biology X. Geostatistical analysis. Eur J Soil Biol 31:173–181

Satchell JE (1971) Earthworms. In: Philipson J (ed) Methods of study in quantitative soil ecology: population. Production and energy flow. Blackwell Scientific Publications, Oxford, pp 107–127

Schmidt O (2001) Appraisal of the electrical octet method for estimating earthworm populations in arable land. Ann Appl Biol 138:231–241

Siegel S, Castellan NJ (eds) (1988) Non parametric statistics for the behavioral sciences. McGraw-Hill, New York

Sims RW, Gerard BM (eds) (1999) Earthworms. Published for The Linnean Society of London and The Estuarine and Coastal Sciences Association by Field Studies Council, Shrewsbury

Stein A, Ettema C (2003) An overview of spatial sampling procedures and experimental design of spatial studies for ecosystem comparisons. Agric Ecosyst Environ 94:31–47

Valckx J, Cockx L, Wauters J, Van Meirvenne M, Govers G, Hermy M, Muys B (2009) Within-field spatial distribution of earthworm populations related to species interactions and soil apparent electrical conductivity. Appl Soil Ecol 41:315–328

Whalen JK, Costa C (2003) Linking spatio-temporal dynamics of earthworm populations to nutrient cycling in temperate agricultural and forest ecosystems. Pedobiologia 47:801–806

Yu JC, Jiang ZT, Li R, Chan SM (2003) Chemical composition of the essential oils of *Brassica juncea* (L.) Coss. grown in different regions, Hebei, Shanxi and Shandong, of China. J Food Drug Anal 11:22–26

Zaborski ER (2003) Allyl isothiocyanate: an alternative chemical expellant for sampling earthworms. Appl Soil Ecol 22:87–95

Zasada IA, Ferris H (2004) Nematode suppression with brassicaceous amendments: application based upon glucosinolate profiles. Soil Biol Biochem 36:1017–1024