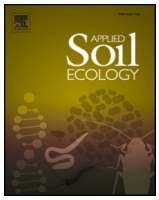
Applied Soil Ecology 206 (2025) 105771

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil



A density-based method to objectively quantify earthworm activity

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ARTICLE INFO

Keywords:
Earthworm activity
Density separation
Universal method
Estivation
Soil ingestion

ABSTRACT

Earthworms are among the most important soil fauna. To exert effects on soils, they need to be active. Earthworm presence is often used as an indicator for activity, but this is not always reliable as earthworms may survive long periods of inactivity or even estivation. Other direct, reliable, and objective measurements for earthworm activity are lacking. Here, we present a novel earthworm activity measurement, based on body-density difference between actively feeding and inactive earthworms. The underlying principle is that active earthworms have a higher density as their gut is filled with soil particles, while inactive earthworms generally have an empty gut. The method therefore separates inactive earthworms from active ones by flotation. To achieve separation, a 1.08 g cm^{-3} sucrose solution is used. Since it is well established that earthworm activity is reduced in dry soil conditions, we set up a soil moisture gradient experiment to gain a range in earthworm activity. We tested our method on four common European earthworms (endogeic species *Allolobophora chlorotica* [Savigny] and *Aporrectodea caliginosa* [Savigny], anecic species *Aporrectodea longa* [Ude] and epigeic species *Lumbricus rubellus* [Hoffmeister]) in an experiment with three soil moisture levels (84, 126 and 168 mL kg^{-1}) using a sandy topsoil. As additional proxy for earthworm activity, estivation was visually recorded. We found a high inter-method correlation between density-based and visually estimated earthworm activity, regardless of earthworm species and moisture treatment. Furthermore, our novel method detected inactive individuals of *L. rubellus*, a species without the ability of estivation. We demonstrated that our density-based method allows for easy and quick quantification of active earthworms. This method offers clear advantages over visual assessments of estivation or cast production, in particular objectivity and applicability to a wider range of species, including those that do not enter estivation.

1. Introduction

Earthworms are among the most important soil fauna because of their strong impact on soil physical, chemical and biological functioning (Vidal et al., 2023). It is estimated that all topsoil in temperate regions passes through earthworm guts every 10–20 years (Edwards and Bohlen, 1996). Through this feeding activity, earthworms create burrows and aerate the soil (Kim et al., 2017; Arrázola-Vásquez et al., 2022), while by foraging on organic residues and soil particles they enhance organic matter decomposition (Lubbers et al., 2017; Barthod et al., 2020) and mineral weathering (Suzuki et al., 2003; Bityutskii et al., 2016). Their production of casts promotes soil aggregate formation (Pulleman et al., 2005; Lubbers et al., 2017) and increases plant-available nutrient pools (Van Groenigen et al., 2014; Ros et al., 2017). Because of these positive effects on soil fertility, their activity is estimated to be responsible for 6.5 % of global grain production (Fonte et al., 2023).

Despite appreciation of the importance of earthworms, it is often overlooked that earthworm *activity* rather than earthworm *presence* relates directly to these soil benefits. Earthworm activity may vary considerably over space and time because of environmental factors, in particular soil moisture content and temperature (Edwards, 2004; Eggleton et al., 2009). For example, in temperate regions earthworm activity often follows a seasonal pattern. Activity of earthworms is remarkably reduced during winter, when the soil temperature drops below 0–5 °C (Daugbjerg, 1988), or during a dry summer, when the soil moisture tension is below wilting point (Curry, 2004).

Decreased earthworm population activity often coincides with a decrease in population size (Bayranvand et al., 2017; Singh et al., 2021),

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https://doi.org/10.1016/j.apsoil.2024.105771
Received 27 May 2024; Received in revised form 22 November 2024; Accepted 25 November 2024
Available online 2 December 2024

but this relation is not constant among all earthworm species (Eggleton et al., 2009; Singh et al., 2021). Individuals of some species with short life-cycle and fast maturation (mostly epigeic species) may die when soil conditions turn unfavourable, with survival of the species ensured by the production of frost- and drought tolerant cocoons (Holmstrup and Overgaard, 2007). Individuals of other species, with a longer life-cycle and slower maturation (mostly anecic and endogeic species) may migrate deeper into the soil (Gerard, 1967; Holmstrup and Zachariassen, 1996), and enter inactivity. This may be a quiescent phase which is immediately reversible when soil conditions turn favourable again, or a seasonal, physiologically determined inactivity of diapause (Edwards and Arancon, 2022; Lavelle, 1988), often referred to as (a)estivation (e.g., Eggleton et al., 2009; Holmstrup et al., 2016). Individuals entering either quiescence or estivation empty their gut system (Edwards and Arancon, 2022; Gerard, 1967), coil up, and cover their body with mucus and egested gut content (Holmstrup and Zachariassen, 1996; Holmstrup et al., 2016).

Additional unfavourable soil conditions, imposed either intentionally or inadvertently, may limit earthworm activity both in nature and in experiments. Such conditions can include low food availability (Lubbers et al., 2017), excessively high earthworm population density (Reinecke and Viljoen, 1990), application of certain irritating compounds (Natalio et al., 2021), or highly compacted soil (Söchtig and Larink, 1992). However, earthworm activity itself is rarely addressed in experiments, with researchers typically using earthworm presence (e.g., Scullion and Malik, 2000) or population size and biomass (e.g., Bayranvand et al., 2017; Singh et al., 2021; Natalio et al., 2021) as a proxy for earthworm activity. Such proxies are clearly not accurate for revealing the activity of species which may enter quiescence or estivation.

A relatively small number of studies use visual methods to estimate earthworm feeding activity, for example by counting earthworms in estivation coils (Daugbjerg, 1988; Holmstrup et al., 2016), or by counting traces, e.g., surface casts (Gerard, 1967; Chevallier et al., 2006; Moos et al., 2016; Spiegel et al., 2018). However, both methods have a limited applicability. For instance, epigeic species often do not have the ability of quiescence/estivation, and surface casting is not typical for endogeic species. Moreover, the accuracy and objectivity of such visual estimations are being questioned across disciplines (e.g., Carlier et al., 2020; Yip et al., 2020).

We propose an alternative, quantitative and objective way of measuring earthworm feeding activity. Soil dwelling earthworms inhabiting mineral soils have a common characteristic in their diet: they ingest food with a density that is approximately 2.5 fold higher than their own body density. Invertebrate body tissues range in density between 0.91 and 1.11 g cm^{-3} (Hasgall et al., 2022), compared to a density of soil particles of around 2.65 g cm^{-3} (Schjønning et al., 2017). Therefore, an earthworm with an empty alimentary canal, either due to being in an estivation phase or in any other non-feeding inactive period, can be separated from active earthworms based on density differences. This may apply for all earthworm species across ecological groups, which consume mineral particles to some extent.

Here we present such a density separation method for soil dwelling earthworms. Due to lack of data on earthworm body tissue composition and body density, we empirically tested a separation solution on four common earthworm species from different ecological groups (endogeic *Allolobophora chlorotica* [Savigny] and *Aporrectodea caliginosa* [Savigny], anecic *Aporrectodea longa* [Ude] and epigeic *Lumbricus rubellus* [Hoffmeister]). We tested our method in an experiment with soil moisture variation acting as a control on earthworm activity (Gerard, 1967; Daugbjerg, 1988). The novel density-based method was compared to a traditional method to estimate earthworm activity through visual assessment of estivating worms. In addition, we measured earthworm population size and biomass. Our hypotheses were that (I) our novel method separates active earthworms from inactive ones, revealed by a high correlation with the visual assessment of estivating worms; but (II) that the density-based method would have a broader applicability, since it may also detect inactivity of a species such as L. *rubellus*, which does not have the ability to enter estivation. We also hypothesized (III) that our novel method correctly works in finding the lowest activity in the driest soil (a well-known effect), but neither population size nor population biomass change would relate to earthworm activity at the different soil moisture levels.

2. Materials and methods

2.1. Principles of the density-based activity measure

When preparing a separation solution for our density-based method, we aimed to make a solution slightly denser than an earthworm body. Since we could not find appropriate data of earthworm body density, we used an empirically calibrated solution. Both because of concern for earthworm well-being and to prevent potential gut content excretion of earthworms due to irritation, we aimed for the least harmful solute. We decided to use sucrose, an easily available, non-irritant compound. Similarly, we aimed for the least concentrated solution, to limit the osmotic stress of earthworms. We diluted 20 g of sucrose in 100 mL water, resulting in a solution density of 1.08 g cm^{-3}, which was around the upper limit in density mentioned for invertebrate body tissues (Hasgall et al., 2022). Before the actual experiment, we successfully tested the method on earthworms, which had an empty gut system after two days of gut voiding (data not shown here).

2.2. Experimental setup

Since it is well established that earthworm activity is reduced in dry soil conditions (Gerard, 1967; Daugbjerg, 1988), we set up a soil moisture-gradient experiment to gain a range in earthworm activity for our activity measurements. We used plastic containers (7 cm diameter, 14.5 cm height) as microcosms. Sandy topsoil classified as typic endoaquoll (Soil Survey Staff, 1999) was collected from the Droevendaal experimental farm at the Wageningen UR campus (86 % sand, pH 5.24, 1.8 % organic matter). Three moisture treatments (84, 126 and 168 mL kg^{-1} dry soil) were established, which represented dry, mesic and wet soil for earthworms. Microcosms were filled with 400 g of dry-weight based soil and compressed to a bulk density of 1.20 g cm^{-3}. Additionally, air-dried Poplar (*Populus* sp.) leaves were milled with a plant-material grinder mill, with a 10 mm sieve inlet, and 6.0 g from this organic residue were added to each of the microcosms. 4.0 g was mixed into the top 5 cm layer of the soil, while 2.0 g was left on the soil surface, to provide an easily accessible food source for all ecological types of earthworms.

Four common European earthworm species representing members of the main ecological groups (*A. longa*, *A. caliginosa* and *A. chlorotica*, *L. rubellus*) were collected from a park area in Wageningen (51°58′50″N 5°39′33″E) on the 24th of March 2022. Adult and adult-sized juvenile earthworms were collected after soil vibration with a gardening fork, except *A. chlorotica*, which was hand sorted from topsoil under poplar leaf litter. All these species, except L. *rubellus*, are known to have the ability of estivation (Eggleton et al., 2009; Holmstrup et al., 2016).

Earthworm population biomass was measured for each microcosm at the start of the experiment, after two days of gut voiding (Dalby et al., 1996), performed right after earthworm collection. Average fresh individual weights were 0.27 (± 0.04), 0.30 (± 0.03), 0.77 (± 0.11) and 1.18 (± 0.18) g, for *A. caliginosa*, *A. chlorotica*, *L. rubellus* and *A. longa*, respectively. Since adults of these four earthworm species differ considerably in size, fewer individuals were introduced per microcosm of the bigger species. Thus, six individuals were used from *A. caliginosa* and *A. chlorotica*, four from L. *rubellus* and three from *A. longa*. The experiment had a completely randomized set up, with three moisture contents, four earthworm treatments and seven replicates, totalling to 84 microcosms. To allow aeration but prevent earthworm escape, microcosms were closed with pieces of black polyester cloth. The

incubation lasted for 14 days in a dark temperature-controlled room (15.5 ± 1.0 °C).

After the incubation, microcosms were harvested. Earthworms were gently hand-sorted from the soils. During sorting, the number of alive earthworm individuals, as well as individuals found in quiescence/estivation was recorded (individuals coiled up to a spherical cell, see e.g., Edwards and Arancon, 2022; Holmstrup et al., 2016). While dry soil conditions most likely triggered quiescence, we were not able to distinguish between those two stages of inactivity. For the sake of simplicity, we will hereafter exclusively use the term estivation, which is often used to cover any type of inactivity stages (Edwards and Arancon, 2022). For the density-based activity measurement (see also Supplementary video), all earthworms found in a microcosm were placed in a plastic sieve and were gently washed to remove any soil particles and mucus adhered to their skin. Afterwards, excess water was removed from their bodies with a paper towel to prevent major dilution of the sucrose solution. Earthworms were subsequently placed in a graduated cylinder, and 500 mL from the sucrose solution was gently poured over them. After a few seconds of settling time, sinking earthworms were classified as 'active', and floating earthworms as 'inactive'. After the separation, earthworms were filtered out with the plastic sieve, washed again to remove remnants of the sucrose solution and placed in gut-voiding tubes for two days, after which they were weighed. Unfortunately, a few earthworm individuals managed to escape through the ventilation holes of the gut voiding tube. Those replicates (n = 4) were excluded from biomass change analysis.

2.3. Calculations and statistical analysis

To allow comparison between species with differing starting populations and biomasses per microcosm, all measured variables were transformed to percentages. Thus, the following variables were used: *survival* (no. of alive earthworms / no. of introduced earthworms), *density-based activity* (no. of active earthworms in separation solution / no. of alive earthworms), *visual-based activity* ([no. of alive earthworms − no. of earthworms in estivation] / no. of alive earthworms) and *biomass change* ([biomass after the incubation − biomass before the incubation] / biomass before the incubation).

The statistical programming language R (version 4.3.0, R Core Team, 2022) was used for statistical analysis under the R studio environment. In order to address inter-method reliability between visual-based and density-based earthworm activity, a concordance correlation coefficient analysis (CCC; Lawrence and Lin, 1989) was performed (package *DescTools*, Signorell, 2023). Since estivation could not be estimated for L. *rubellus*, this species was excluded from the CCC analysis. We also used non-parametric Wilcoxon tests to compare the two activity measures for each species and moisture treatment combination.

To test our hypothesis that the driest soil is the least favourable for earthworms, all variables were compared between soil moisture treatments. Kruskal-Wallis non-parametric tests with subsequent post-hoc Dunn tests (package FSA, Ogle et al., 2021) were performed on two levels, across all species and within every species separately.

Finally, we used logistic regression models to identify whether earthworm survival or earthworm weight change predicts visual-based or density-based earthworm activity. We decided on logistic regression models rather than correlation analyses since earthworm activity and earthworm survival are discrete variables, while the change in earthworm weight is a continuous variable. We set a threshold for earthworm activity at 100 %, considering values equal to this threshold as maximal earthworm activity and values below this threshold as inactivity of earthworms is occurring. For these logistic regression models, all relevant studied earthworm species were handled together, except when predicting density-based activity, where correlations were calculated both including and excluding L. *rubellus*. Significance level was set at $p < 0.05$ for all statistical analyses.

3. Results

Survival of earthworms was high during the experiment, since only 6 individuals of L. *rubellus* died (1.5 % of all earthworms used). We counted 43 earthworm individuals in estivation coil and measured 41 (43 when including L. *rubellus*) earthworm individuals as inactive with the density-based method. Thus, average activity based on visual observation of estivation was 87.0 % and activity based on density separation was 87.3 % (89.9 %, including *L. rubellus*). There was a high agreement between the two activity determinations (CCC = 0.795, Fig. 1A). We observed no significant difference between visual-based and density-based activity ($p > 0.05$, Wilcoxon test) in any combination of earthworm species and moisture treatments (Table 1).

Across species, both visual-based and density-based earthworm activity were on average (the latter only with L. *rubellus*) significantly ($p < 0.05$, Kruskal-Wallis test with post-hoc Dunn test) lower at the dry treatment (63.5 % and 71.4 %, respectively) compared to mesic (98.4 % resp. 99.4 %) or wet (99.2 % resp. 98.8 %) treatments. Similar to earthworm activity, biomass change was significantly ($p < 0.05$) lower for the dry treatment, with average biomass reduction (−10.5 %) compared to average biomass gain in the mesic (0.4 %) and wet treatments (5.4 %). Earthworm survival was not affected significantly by moisture treatments. When variables were analysed by earthworm species separately, we observed similar significant ($p < 0.05$) results (Table 1), except that the density-based activity of L. *rubellus* did not differ significantly between moisture treatments.

Due to marginal mortality, earthworm survival was not a significant predictor of earthworm activity in any logistic regression model. However, biomass change was a significant predictor for both visual-based activity ($p = 0.002$) and density-based activity ($p < 0.001$; Supplementary Fig. S1), but only in case L. *rubellus* was excluded. In microcosms where earthworm biomass decreased, more inactive earthworms were found (Fig. 1B, Supplementary Fig. S2).

4. Discussion

We confirmed our first hypothesis, since the novel density-based earthworm activity measure was able to separate active and inactive earthworms given the high agreement with the visual assessment of estivating worms (Fig. 1A). Furthermore, our observations confirmed that sucrose is a harmless separation solution: there was no gut-content excretion, and earthworms were lively even after gut-voiding for two days, after the exposure to the density separation solution.

However, there are differences in the resolution of the density-based method and the visual activity estimation. While the average density-based and visual-based activity of *A. chlorotica*, *A. caliginosa* and *A. longa* for the different moisture treatments were not statistically different (Table 1), there were certainly differences between these measures within the replicates (Fig. 1A). There may be several reasons for density-based earthworm activity being lower than visual-based activity. It could be that some earthworm individuals were preparing for estivation thus emptied their guts, but had not yet coiled up. It is also possible that earthworms have longer periods during which they reduce feeding, but still burrow for exploration. Perreault and Whalen (2006) suggested this too, by observing higher burrowing activity, but less cast production and weight loss of earthworms in dryer soil conditions. Explanations for the opposite case, where density-based activity was higher than visual-based activity, may include incomplete excretion of gut content by a few earthworm individuals, or remarkable dehydration of earthworms in diapause (Holmstrup et al., 2016), which could increase body density. Of course, it also cannot be ruled out that the visual estimation was simply inaccurate. It may be that due to the disturbance of the destructive harvest, despite having been as quick and gentle as possible, few earthworms left the estivating spherical cell, while other active earthworms may coil up due to the same disturbance.

While our method is yet intended to test the feeding activity of soil

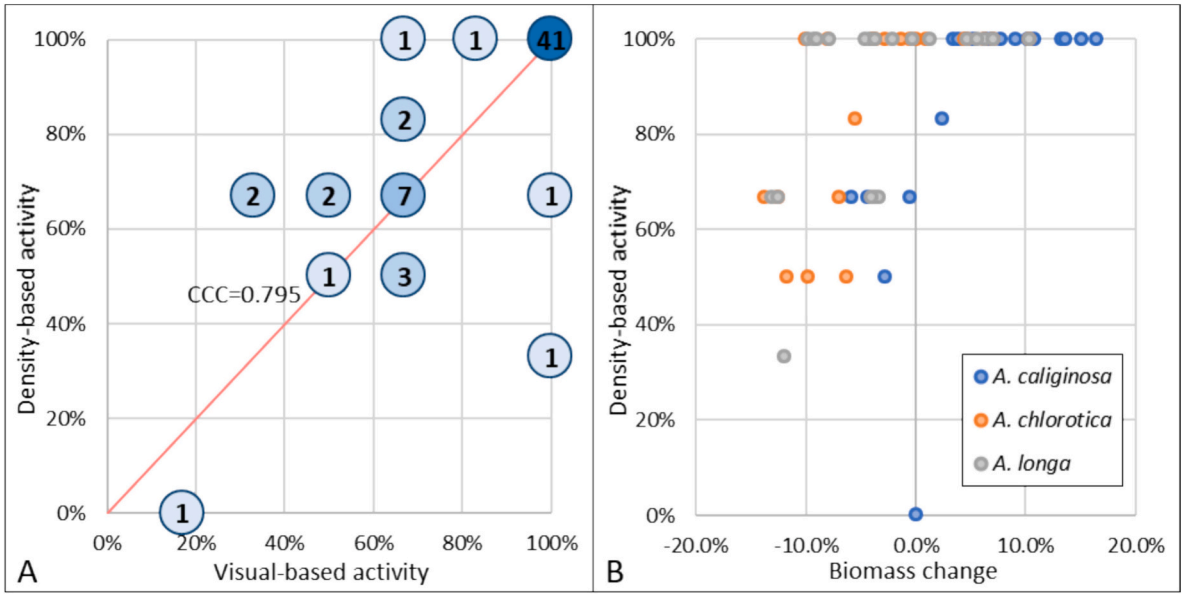

Fig. 1. (A) Relation between visual-based and density-based earthworm activity for the species for which estivation rate could be investigated (*A. chlorotica*, *A. caliginosa* and *A. longa*). Numbers in dots (as well as the intensity of dot-colour) represent the amount of overlapping observations. CCC = concordance correlation coefficient. (B) Relation between earthworm biomass change and density-based activity for the same three earthworm species.

Table 1
Earthworm survival, visual-based and density-based activity and population biomass change of the four studied earthworm species per moisture treatments. For percentage calculations, see Section 2. Materials and methods. Values between parentheses are standard errors (n = 7, except n = 6 for *A. caliginosa* * Dry and *L. rubellus* * Mesic and n = 5 for *L. rubellus* * Wet for biomass change). Letters in superscript indicate significant ($p < 0.05$, Kruskal-Wallis test with post-hoc Dunn test) differences between soil moisture treatments for a given earthworm species. The lack of asterisks indicates no significant ($p > 0.05$, Wilcoxon test) differences between the visual-based and density-based activity for each separate treatment combination.

Species	Soil moisture	Survival (%)	Biomass change (%)	Visual-based activity (%)	Density-based activity (%)
A. chlorotica	Wet	100 (±0)	4.8 (±1.3)^{a}	100 (±0)^{a}	100 (±0)^{a}
A. chlorotica	Mesic	100 (±0)	−4.2 (±1.3)^{b}	100 (±0)^{a}	100 (±0)^{a}
A. chlorotica	Dry	100 (±0)	−9.5 (±1.2)^{b}	66.7 (±0)^{b}	61.9 (±4.4)^{b}
A. caliginosa	Wet	100 (±0)	12.7 (±1.0)^{a}	97.6 (±2.2)^{a}	100 (±0)^{a}
A. caliginosa	Mesic	100 (±0)	4.7 (±0.6)^{b}	95.2 (±4.4)^{a}	97.6 (±2.2)^{a}
A. caliginosa	Dry	100 (±0)	−0.9 (±1.8)^{b}	52.4 (±6.2)^{b}	59.5 (±10.6)^{b}
A. longa	Wet	100 (±0)	2.3 (±2.2)	100 (±0)^{a}	95.2 (±4.4)^{a}
A. longa	Mesic	100 (±0)	−3.8 (±2.1)	100 (±0)^{a}	100 (±0)^{a}
A. longa	Dry	100 (±0)	−7.6 (±1.8)	71.4 (±10.5)^{b}	71.4 (±8.0)^{b}
L. rubellus	Wet	96.4 (±3.3)	0.6 (±4.2)^{a,b}	NA	100 (±0)
L. rubellus	Mesic	100 (±0)	5.8 (±1.6)^{a}	NA	100 (±0)
L. rubellus	Dry	82.1 (±8.3)	−22.7 (±9.2)^{b}	NA	92.9 (±4.3)

dwelling earthworms, we included L. *rubellus* in our experiment too, although it is an outlier compared to the other used species. *L. rubellus* cannot estivate (Holmstrup et al., 2016), and it is usually considered a litter-dwelling epigeic species. This species additionally explores the surface layer of the mineral soil and can therefore also be classified as an epi-endogeic earthworm (Bottinelli et al., 2020). Although statistically insignificant, a few individuals of L. *rubellus* were detected as inactive with the density-based measure. While this finding in itself could have been due to some measurement inaccuracy, the fact that these inactive L. *rubellus* individuals were only found in the driest treatment suggest something more meaningful. The density-based method therefore has a broader applicability than visual estivation rate estimation. It seems that even those earthworm species which do not have the strategy to survive unfavourable conditions in estivation, may reduce their feeding activity when soil conditions deteriorate. This may be a mechanism to avoid desiccation, since their body is only exposed from outside to drought stress. However, this hypothesis needs further investigation.

As expected, soils with lowest moisture content affected earthworms the most, indicated by significantly lowest activity but also by average weight loss of all the studied earthworm species (Table 1). There were no significant differences in earthworm activity between mesic and wet treatments, while biomass gain was significantly higher for the wet treatment (especially for the endogeic species; Table 1). This suggests that earthworm wellbeing increases gradually with increasing soil moisture content, while inactivity is more triggered by a certain limit in soil moisture (Curry, 2004).

The marginal mortality and resulting lack of relation with activity shows the inaccuracy of population size as an earthworm feeding activity measure, at least for short-term studies. At the same time, the significant predictive ability of earthworm biomass change on inactivity occurrence was unexpected. It appears that weight loss is coupled to reduced feeding activity of earthworms, which was suggested by e.g., Natalio et al. (2021), but not uniformly (Fig. 1B) and not for every species (Supplementary Fig. S2). The lower predictive ability of weight change on activity when L. *rubellus* was included in the logistic regression model suggests an accuracy limitation to certain species.

Is our new method superior to other methods? In Table 2 we compared earthworm-activity estimating methods using various criteria. A clear difference between this novel density-based measure and the other activity estimates is that it reveals up-to-date earthworm

Table 2
Comparison of methods used for addressing earthworm activity.

	Measuring change in earthworm population size or biomass	Estimation of earthworm structures (casts, burrows, etc.)	Estimation of estivation rate	Proposed density-based measure
Gained information	Cumulative indicator of earthworm-population wellbeing, not so much of activity.	Rough, cumulative earthworm activity estimate in earthworm-population level on a given trace of earthworm activity.	Current earthworm population inactivity in earthworm-individual level for species with ability for estivation.	Current earthworm population feeding activity in earthworm-individual level.
Applicability for species	Used for most species	Used for most species	Only for species with ability for quiescence or estivation.	Could be widespread, but have not been verified for entirely litter dwelling epigeic earthworm species or compost worms.
Objectivity	Quantitative	Semi-quantitative (e.g., visual cast counting) to quantitative (e.g., burrow length measured with X-ray tomography)	Semi-quantitative	Quantitative
Speed of analysis	Fast, dependent on the population size.	Moderate to slow, dependent on the addressed activity trace and used method.	Moderate, dependent on the population size.	Fast, in case population size is addressed.
Sampling	Destructive sampling	Destructive sampling, except addressing surface casts	Destructive sampling	Destructive sampling
Special considerations	Mortality of worms matters a lot in interpretation of earthworm biomass data.	Surface casts may be disturbed, not all species cast on surface. Burrowing activity may not correlate with feeding activity.	Needs to be done immediately during destructive sampling, as earthworms tend to leave estivation after disturbance.	Needs to be done immediately after destructive sampling, as active earthworms would otherwise start to void their gut content.
Equipment	Separate containers for each experimental unit, paper towel, scale	Dependent on addressed activity trace and chosen method	Nothing	Fractionation solution, sieve, water, paper towel, tall beaker
Handling of high earthworm population	Possible	Possible	Difficult	Possible
Ethics	Gut-voiding may be harmful	X-ray may be harmful	Non-harmful	Non-harmful
Example of studies, which used as a proxy for earthworm activity	E.g., Bayranvand et al., 2017; Singh et al., 2021; Natalio et al., 2021	E.g., Gerard, 1967; Pulleman et al., 2005; Chevallier et al., 2006; Perreault and Whalen, 2006; Capowiez et al., 2021	E.g., Daugbjerg, 1988; Holmstrup et al., 2016	–

activity at the individual earthworm level. Only estivation counting shares this property, although this method has been mostly used for physiological studies of estivating earthworms (Holmstrup et al., 2016), rather than estimating earthworm population activity. All other activity estimates are based on comparing traces connected to the activity of a whole earthworm community, such as casts (Gerard, 1967; Chevallier et al., 2006; Perreault and Whalen, 2006), burrows (Perreault and Whalen, 2006; Capowiez et al., 2021) or all other worm-affected parts of the soil (Pulleman et al., 2005). Since these traces are often semi-persistent, they might not reflect the current earthworm activity.

Another difference between our method and the other approaches is objectivity. This method uses an exact measure to quantify activity, while most other approaches use at least in some extent visual assessment, which could be biased by surveyors. An exception is measuring burrowing length with X-ray tomography (Capowiez et al., 2021). However, in addition to the fact that this is a very expensive and time-consuming option, there is some evidence that burrowing activity does not always relate to feeding activity of worms (Perreault and Whalen, 2006; Capowiez et al., 2021).

There are differences in the execution simplicity of these methods too. Our density-based measure is easily implemented without much extra effort in experiments in which the earthworm population size is addressed anyway. Even though counting estivation could be combined with counting alive earthworms, it requires much extra time and carefulness during harvest. Other activity measures at the community level (e.g., cast counting) require additional measurements or estimations.

It is worth noting that all activity measures, except surface cast counting, can only be performed after destructive sampling. Thus, in case earthworm activity is needed to be monitored during the experiment, cast counting may be the only option. This method has serious drawbacks though: surface objects and watering or raining may obstruct counting. Furthermore, continuous monitoring of casting using this method is certainly inaccurate for endogeic species which may often cast beneath the soil surface (Capowiez et al., 2021), or for anecics, which may cast in the same place forming middens (Rossi and Nuutinen, 2004).

We conclude that this novel density-based method is a useful additional measure to quantify earthworm activity. Density separation offers a quick and easy way to address earthworm feeding activity for species ingesting mineral particles. Such an individual-based measure may be best to study relations between soil properties and earthworm activity. As a next step, our method needs to be tested with a wider number of species. Given the few, but promising inactivity records of L. *rubellus*, it is especially important to further verify the usability of our approach for epigeic species. Since epigeic earthworms usually lack the ability for estivation, our density-based method may be the only way to measure activity of these species in an individual-based manner. It is worth noting, that the density of the separation solution is also open for calibration, in case active specimens of other species, for instance, epigeics or anecics would have a different body density due to a lower contribution of mineral particles to their diet. Furthermore, future studies could explore the applicability of this method to additional environmental and anthropogenic factors known to influence earthworm activity, such as soil temperature and agricultural management practices.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2024.105771.

CRediT authorship contribution statement

Péter Garamszegi: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Tullia Calogiuri:** Writing – review & editing, Supervision, Methodology, Data curation, Conceptualization. **Mathilde Hagens:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Alix Vidal:** Writing – review & editing, Supervision, Methodology, Investigation. **Jan Willem Van Groenigen:** Writing –

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank Unifarm at Wageningen University & Research for providing the experimental soil and Zhongchen Yang for recording the supplementary video. PG acknowledges funding from the Swedish Research Council project led by Eveline Krab (grant no. 2021-04458) during the writing of the manuscript. TC acknowledges funding from the Horizon 2020 project Bio-Accelerated Mineral Weathering (BAM!) of the European Union (grant agreement no. 964545).

Data availability

Data will be made available on request.

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