**Ecological groups and isotopic niches of earthworms**

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**Abstract**

Earthworms are generally categorized into three ecological groups. This categorization and its two refined versions have been widely used in studies focusing on earthworm community structure, biological invasions, and earthworm effects on vegetation, soil properties, carbon and nitrogen cycling, and other ecosystem functions. We revisited this categorization system by analyzing the stable isotopes of carbon and nitrogen (13C and 15N) from 10 earthworm species commonly found in arable fields and deciduous forests in temperate North America. We showed that up to nine isotopic niches utilized by earthworms can be distinguished in the two-dimensional space delimited by δ13C and δ15N. Rather than forming distinct categories, isotopic niches of different ecological groups form a continuum, ranging from species feeding on leaf litter to those feeding on highly processed soil organic matter. Along this continuum, species of the same ecological group can exhibit considerable isotopic niche differentiation and resource partitioning. These results, together with field observations on native Lumbricidae in Europe, suggest that, despite their convenience, there are limitations to the commonly used ecological categories. We propose to incorporate stable isotopes as functional traits in studying earthworm ecology and the linkage between earthworm communities and ecosystem functions. Moreover, to fill existing knowledge gaps, the isotopic approach needs to be further expanded to native earthworm communities around the world, particularly outside Western Europe and North America, and to earthworm families and major taxonomic groups other than Lumbricidae, such as Glossoscolecidae, Acanthodrilidae, and Megascolecidae.

**Keywords**: Earthworms, Stable isotopes, Functional groups, Epigeic, Anecic, Endogeic

**1. Introduction**

Guilds, ecological groups, and functional groups are commonly used concepts in food web modeling and in studies attempting to link community structures to ecosystem functions (Wilson, 1999; Blondel, 2003; Voigt et al., 2007). They provide a proxy without detailed knowledge on the taxonomic identity and ecology of individual species. In studies focusing on the soil ecosystem, this is frequently the only feasible approach as the large number of species of soil fauna, most of which are unknown to science, makes incorporating species identities into models practically infeasible. Applied soil ecology projects, such as assessment of effects of land management, disturbances and recovery, or efficiency of ecosystem restoration, may include soil fauna surveys (e.g. Frouz et al., 2013; Gongalsky and Persson, 2013). Due to lack of time, taxonomic expertise, or other resources, or because an exact species list is not the focal component of the study, researchers may report on larger taxonomic or ecological groups. This is true not only for microscopic animals, such as nematodes and collembolans, but also for organisms that are relatively large and perceived as well-studied, such as earthworms.

Earthworms are the dominant group of soil fauna in many temperate and tropical ecosystems. Their feeding and burrowing lead to translocation and transformation of detritus and soil organic matter, affect soil microbial communities, and change resources available to other soil animals (Frelich et al., 2019; Ferlian et al., 2020; Chang et al., 2021). They are often considered “ecosystem engineers” as their presence fundamentally changes the soil habitat, and has profound impacts on the understory vegetation, leaf litter layer, soil properties, nutrient dynamics, and soil C and N cycles (Craven et al., 2017; Frelich et al., 2019; Ferlian et al., 2020; Chang et al., 2021).

Earthworms are generally categorized into three main ecological groups—epigeic, endogeic, and anecic—based on their morphology, location in the soil profile, and feeding habits (Bouché, 1977). Epigeic species are litter feeders living in the litter-soil interface; endogeic species are soil feeders living in the soil; anecic species are large-bodied litter feeders that live in permanent vertical burrows deep in the soil (Brown, 1995; Curry and Schmidt, 2007). Although researchers quite often credited and therefore cited Bouché (1972, 1977) for the three terms, the concepts of these ecological categories had taken shape almost a century before Bouché (Örley, 1885), and their definitions have since been modified by various authors and have evolved considerably (Lavelle, 1981; Lee, 1985).

While rarely acknowledged by modern researchers, ecological categorization of earthworms similar to the system used nowadays was first proposed by Örley (1885), who distinguished three groups: Large-bodied species penetrating to 90–120 cm deep in the soil of forests and meadows; smaller species living in wood and other vegetable matter; species inhabiting the clayey soil and mud of riparian banks and standing waters. Although Örley (1885) did not name them, the three groups are consistent with the modern concept of anecic, epigeic, and endogeic species, respectively. Similar categories for the New Zealandian Megascolecidae were presented by Lee (1959), who distinguished litter species (small and heavily pigmented), topsoil species (large, pigmented, and living in permanent burrows), and subsoil species (large, unpigmented, and living in the deeper soil layers), and by Perel (1975), who recognized two main life forms (ecological groups), one feeding on the soil surface and the other feeding in the soil. Perel (1975) further divided these two main groups into subgroups based upon their vertical distribution in the soil. The first group, i.e. those feeding on the soil surface, were categorized into (1) surface living species, (2) transitory species active between the surface and the upper soil layer, and (3) deep burrowing species. The second group, i.e. species feeding in the soil, were categorized into three subgroups as well: living in the (1) upper, (2) middle, and (3) deeper soil layers.​​

With 50 years of research on earthworm taxonomy, evolution, life history, physiology, and ecology after Bouché’s 1972 book, we now have a better understanding on the spectrum of the ecological diversity of these organisms (Neilson et al., 2000; Curry and Schmidt, 2007; Zicsi et al., 2011), partially aided by studies focusing on invasive earthworms and their ecological impacts (Szlavecz et al., 2011, 2018; Chang et al., 2017, 2021; Craven et al., 2017; Taheri et al., 2018; Frelich et al., 2019; Ferlian et al., 2020). Currently, two different systems of earthworm ecological categorizations are most frequently used by taxonomists and ecologists: (1) Bouché’s system (Bouché, 1972, 1977) and (2) Perel and Lavelle’s system (Perel, 1975; Lavelle, 1981). Bouché’s (1972, 1977) system is composed of three main categories—epigeic, endogeic, and anecic—and four intermediate categories—epi-anecic, endo-anecic, epi-endogeic, and intermediate. Lavelle (1981) took Bouché’s (1972, 1977) three main categories and further divided the endogeic group into three categories: polyhumic endogeic, mesohumic endogeic, and oligohumic endogeic (Fig. 1). Through years of use, the term epi-endogeic was also included by subsequent researchers into their vocabulary to describe species that not only live in the leaf litter but also spend a considerable amount of time in the surface soil (Fig. 1). Coincidentally, these six categories completely overlap with the aforementioned six subgroups proposed by Perel (1975). Both Bouché’s system and Perel and Lavelle’s system are widely adopted by earthworm researchers (Brown, 1995; Bottinelli et al., 2020; Bottinelli and Capowiez, 2021), likely for being relatively comprehensible and easy to use.

Regardless of which system was adopted, a simplified version containing only the three main categories, epigeic, endogeic, and anecic (e.g. Edwards and Bohlen, 1996; Bohlen et al., 2004; Briones and Alvarez-Oteroet, 2018; Potapov et al., 2022), has been widely used to investigate how earthworms, particularly invasive species, affect the structure and function of ecosystems (Brown, 1995; Bottinelli et al., 2020; Bottinelli and Capowiez, 2021). Such studies have been summarized in meta-analyses focusing on plant growth (van Groenigen et al., 2014; Xiao et al., 2018), soil nutrient and toxic elements (van Groenigen et al., 2019; Ferlian et al., 2020; Sizmur and Richardson, 2020), soil faunal and microbial biodiversity (Ferlian et al., 2018), plant communities (Craven et al., 2017), and greenhouse gas emission (Lubbers et al., 2013). In most of these studies, the categories epigeic, endogeic, and anecic are considered as “ecological groups”, while in others, they are considered as “guilds” (van Groenigen et al., 2019) or “functional groups” (Ferlian et al., 2018). In studies focusing on ecosystem functions, different species in the same group have sometimes been reported having inconsistent effects on the targeted ecosystem properties (e.g. Chang et al., 2016a, b), calling into question whether these ecological categories are meaningful proxies for functional entities.

Community ecologists have long acknowledged that guilds, ecological groups, and functional groups encompass different concepts, yet in practice, these terms have been frequently used interchangeably (see Blondel, 2003 for a detailed discussion). In earthworms, even when “ecological groups” was used, as in most aforementioned studies, it was treated implicitly as synonymous to functional groups, as the categorization was used to infer ecological impacts on other organisms or ecosystem functions, rather than niche partitioning between groups or interspecific interactions within groups. However, given that ecological groups are, by definition, not synonymous to functional groups, the inconsistent results researchers have observed so far should not be surprising. In earthworm surveys, species are often classified into one or another category based upon their color or where they were found in the soil profile, without detailed knowledge about their resource uses and mixing activities. Zicsi et al. (2011) highlighted this mismatch in a series of experiments using 30 Lumbricidae species from Europe, of which 21 were endemic. If species in the same ecological category already differ in litter feeding and soil mixing, the two fundamental characteristics that define the three main ecological categories, how could we expect these species to have similar influence on ecosystem functions?

In the last decade, several researchers appealed for not treating ecological categories and functional groups as synonyms (Bottinelli and Capowiez, 2021), for strictly adhering to Bouché’s (1972, 1977) original definition of the three main groups and four intermediate groups (Bottinelli et al., 2020), for using species identity instead when investigating ecosystem functions (Chang et al., 2016b), and for redefining or refining these groups (Neilson et al., 2000; Zicsi et al., 2011). Recently, the trait-based approach was used to numerically redefine Bouché’s seven categories based on 13 anatomical and histological morphologies, offering the first quantitative approach to categorizing earthworm ecological groups (Bottinelli et al., 2020). The study concluded that earthworm’s ecological strategies are continuous rather than categorical, and epigeic, endogeic, and anecic are just the three endpoints of this continuous distribution. In addition to morphology, the authors also called for new traits that can be linked to the ecology and behavior of investigated species.

Stable isotopes have been widely used to study the feeding ecology of soil invertebrates, particularly nematodes (Kudrin et al., 2015; Melody et al., 2016), mites (Maraun et al., 2011), proturans (Bluhm et al., 2019), collembolans (Ferlian et al., 2015; Potapov et al., 2021), and earthworms (Neilson et al., 2000; Melody and Schmidt, 2012; Ferlian et al., 2014; Potapov et al., 2019c). This technique is also instrumental in our current understanding on trophic niche partitioning of soil fauna, and on how different groups of soil fauna are involved in belowground processes (Pollierer et al., 2009; Hyodo et al., 2010; Klarner et al., 2014; Potapov et al., 2019a,b). Isotopic studies focusing on earthworms largely confirmed the three main ecological groups (Schmidt et al., 1997, 2004; Scheu and Falca, 2000; Briones et al., 2001; Pollierer et al., 2009; Potapov et al., 2019c), provided evidence for niche differentiation and competition (Melody and Schmidt, 2012; Chang et al., 2016b), and further highlighted the importance of soil microbes in the diet of earthworms (Ferlian et al., 2014; Larsen et al., 2016). However, while stable isotopes, sometimes coupled with other techniques, have been instrumental in redefining feeding groups in collembolans and mites (Maraun et al., 2011; Potapov et al., 2016, 2021), and despite a plethora of studies on the stable isotope ecology of earthworms, this approach has not been applied to meaningfully refine earthworm ecological groups.

The objective of this study is to examine the idea of using the natural abundance of carbon and nitrogen stable isotopes (13C and 15N) to refine the widely used ecological groups of earthworms under the framework outlined by Perel and Lavelle (Perel, 1975; Lavelle, 1981). We predicted that based on the isotopic niches of earthworm species (1) the three main ecological categories would form a continuum in the isotopic space, (2) species within the same ecological category can be divided into subgroups consistent with Perel and Lavelle's groupings based on their isotopic niche positions, and (3) species within the same subgroup can be further categorized as a trophic specialist or a trophic generalist based on their isotopic niche breadths.

**2. Material and Methods**

*2.1 Field sites and plots*

Data for this study were collected from five sites, representing three habitat types: arable field, abandoned arable field, and mature deciduous forest. Data from arable and abandoned arable fields were newly collected, whereas data from the forests have been reported in Chang et al. (2016b). Information about the sites is detailed below.

*2.1.1 Arable field 1 (ARABLE1)*

Arable field 1 is part of the USDA ARS Beltsville Farming System Project (FSP) in Maryland, USA. The project was established in 1996 to evaluate the long-term sustainability of conventional and organic grain crop production. The five cropping systems differ in crop rotation, mode of tillage, fertilizer use and pest control. A total of 68 plots cover 16 ha on the Atlantic Coastal Plain. The dominant soil types are silt loams with an argillic horizon around 20-60 cm depth. Mean annual temperature is 12.8°C and the 30-yr average annual precipitation at the site is 1110 mm, distributed evenly through the year. The field is not irrigated (Cavigelli et al., 2008). Sampling at Arable field 1 was conducted in a no-till plot (plot 306) with a 3-year rotation of corn - soybean - wheat & soybean. Sampling was conducted on November 8th, 2011, a “corn” year, after corn harvest.

*2.1.2 Abandoned arable fields 1 and 2 (ABANDONED1 and ABANDONED2)*

Abandoned arable fields 1 and 2 are located in properties managed by the Smithsonian Environmental Research Center (SERC), Maryland, USA. The SERC abandoned field site is in a self-contained watershed, 13 hectares of which had been in continuous corn agriculture for 34 years since 1978. Agriculture ceased in 2012, and the site was converted to an experimental research forest manipulating tree diversity and functional traits. A total of 75 plots were delineated and planting of tree seedlings started in 2013. We chose nine plots in one location as potential sites for long term monitoring (ABANDONED1). Another two plots were also sampled (ABANDONED2). These two plots, in addition to being spatially separated from ABANDONED1, showed apparent differences in vegetation and land use history. Specifically, C4 (corn) and C3 (soybean) crops were planted here. At the time of the earthworm sampling (May-June 2013), the fields were covered with corn detritus, and early successional herbaceous weeds, and the first growing season of the planted small tree seedlings just started. We consider this habitat an abandoned corn field and the isotopic signatures of the earthworms reflecting those conditions.

*2.1.3 Mature forests 1 and 2 (FOREST1 and FOREST2)*

Mature forests 1 and 2 are located in the Treefall forest stand at SERC, which is a 150-year-old secondary forest dominated by tulip poplar (*Liriodendron tulipifera*), American beech (*Fagus grandifolia*), and oaks (e.g. *Quercus falcata* and *Q. alba*). The stand was sampled in September 2011 and then again in August 2013. In both years, samples were taken from a 25 × 25 m plot. Because of non-overlapping sampling locations, different terrains, and considerably different earthworm species compositions (Chang et al. 2016b), data from the two sampling events were treated separately (i.e., Mature forest 1 and Mature forest 2). See Chang et al. (2016b) for detailed site description and sampling procedures.

*2.2 Sampling*

At ARABLE1, earthworms were collected from four randomly selected 1 × 1 m quadrats 1–5 m away from the south (short) edge of the no-till plot using electroshocking. A 0–15 cm deep core was collected from each quadrat using a soil corer (5 cm diameter), and divided into three 5-cm increments (0–5 cm, 5–10 cm, and 10–15 cm).

At ABANDONED1 and ABANDONED2, five 0.5 × 0.5 m quadrats were designated at the center and the four corners (5 m away from the plot edge) of the newly established plots. Earthworms were collected by slowly applying mustard suspension on the soil surface within the quadrat. Three 0–20 cm deep soil cores were collected around the center quadrat and a randomly selected corner quadrat using a soil corer (3 cm diameter) and divided into the 0–2, 2–5, 5–10, and 10–20 cm deep increments. Samples of the same increment from the same quadrat were mixed to form a composite sample.

At FOREST1 and FOREST2, earthworms were collected from three randomly selected 1 × 1 m quadrats at least 15 m away from each other using electroshocking. Three 0–15 cm deep cores were collected from each quadrat using a soil corer (5 cm diameter), and divided into three 5-cm increments (0–5 cm, 5–10 cm, and 10–15 cm). Samples of the same increment from the same quadrat were analyzed separately.

The collected earthworms were identified to species alive and then stored at -20°C until further processing. A total of 251 earthworm individuals representing 10 species were collected, including 102 specimens from FOREST1 and FOREST2 (Chang et al. 2016b) and 149 newly collected specimens from ARABLE1, ABANDONED1, and ABANDONED2. The 10 species can be categorized into the three commonly used ecological groups: (1) epigeic—*Lumbricus rubellus* Hoffmeister, 1843 and *Metaphire hilgendorfi* (Michaelsen, 1892), (2) endogeic—*Aporrectodea caliginosa* (Savigny, 1826), *Aporrectodea trapezoides* (Dugés, 1828), *Allolobophora chlorotica* (Savigny, 1826), *Diplocardia caroliniana* Eisen, 1899, *Eisenoides lonnbergi* (Michaelsen, 1894), and *Octolasion cyaneum* (Savigny, 1826), and (3) anecic—*Lumbricus friendi* Cognetti, 1904 and *Lumbricus terrestris* Linnaeus, 1758.

*2.3 Sample processing and isotopic analysis*

Earthworm specimens were cleaned with distilled water, dissected to remove gut and gut content, freeze-dried, and homogenized. Soil samples were sieved and homogenized through a 2-mm sieve, oven-dried, and pulverized. The C and N elemental and stable isotope composition of earthworm and soil samples were analyzed at the UC Davis Stable Isotope Facility, Davis, CA, USA using either a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) coupled with an Elementar Vario EL Cube or Micro Cube elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany) or a PDZ Europa ANCA-GSL elemental analyzer (Sercon Ltd., Cheshire, UK). Stable isotope ratios of C and N (13C/12C and 15N/14N) were expressed using the delta (δ) notation following the equation δ13Csample or δ15Nsample = [ Rsample / Rstandard – 1 ] x 1000‰, where Rsample is the ratio (13C/12C or 15N/14N) in the samples, and Rstandard is the isotope ratios in the standards (Pee Dee Belemnite for C and atmospheric nitrogen for N).

*2.4 Data analyses*

The δ13C and δ15N of earthworm samples were standardized within each site by subtracting the differences between plot-level (the plot from which the earthworm individual was collected) and site-level δ13C and δ15N of soil samples at 0-5 cm depth. For the sites ABANDONED1 and ABANDONED2, the isotope values of soil samples at 0-5 cm depth were derived as the weighted average of the isotope values at 0-2 cm and 2-5 cm depth. We used surface soil for standardization as opposed to leaf litter because at the time of earthworm and soil sampling at ARABLE1, ABANDONED1, and ABANDONED2, the litter on the soil surface was not abundant enough to be a reasonable representative of basal resources of the study sites. In contrast, the surface soil hosted isotopic signals that were integrated through different seasons, and provided a better proxy of background isotopic signatures for our standardization purpose.

To examine the isotopic niche breadth of earthworm species, we calculated Bayesian standard ellipse area (SEAB), which represents the core isotopic niche of a population, using the R package SIBER (Jackson et al., 2011). Three Markov Chain Monte Carlo (MCMC) chains were run, each with 30,000 iterations and a burn-in number of 3,000. An inverse Wishart prior was used to initiate the model. Chain convergence was assessed via trace plots and Geweke diagnostics. The 95% high density interval (HDI) was computed to quantify the uncertainty in SEAB estimates. The percentage of overlapping SEAB was also calculated to measure the degree of core niche overlap between species pairs.

To test for the differences in species’ total isotopic niches (including niche position and niche breadth), we further performed pairwise permutational analysis of variance (PERMANOVA) and permutational test for homogeneity of multivariate dispersions (PERMDISP) on the δ13C and δ15N of earthworm species using the R package vegan (Oksanen et al., 2013). PERMANOVA tests the null hypothesis of no difference in the centroid (i.e., niche position) and/or dispersion (i.e., niche breadth) of the species pairs in the isotopic space, whereas PERMDISP specifically tests the null hypothesis of no difference in the dispersion (i.e., niche breadth) of the species pairs in the isotopic space. All analyses were performed in R version 4.1.0 (R Core Team, 2021).

In addition to data from ARABLE1, ABANDONED1, and ABANDONED2, data collected at FOREST1 and FOREST2 previously reported in Chang et al. (2016b) were also analyzed. As isotopic niche sizes and overlaps in the two forest plots have already been reported in Chang et al. (2016b), the same analyses were not repeated here. However, in Chang et al. (2016b), the position and breadth of the total isotopic niches were compared using a different approach. Thus, to ensure that data from the five plots can be compared and synthesized, we re-plotted the data from FOREST1 and FOREST2 following the same standardization procedure as we did for the other three sites, and re-analyzed niche position and niche breadth using PERMANOVA and PERMDISP.

**3. Results**

*3.1 Stable isotope signatures of soil*

δ13C values of soil samples were lower at the forest sites (FOREST1 and FOREST2) than at the arable/abandoned field sites (ARABLE1, ABANDONED1, and ABANDONED2) (Fig. 2), and had the highest C4 signatures at ABANDONED1. The two forest sites were similar to each other and showed a depth profile of increasing δ13C values in deeper soils. The two abandoned field sites were distinct, with ABANDONED1 showing higher δ13C values than ABANDONED2. Additionally, δ13C values at ABANDONED2 increased with soil depth, similar to those in the forests, whereas δ13C values at ABANDONED1 were lowest in the deepest soil (10-20 cm) followed by the surface soil (0-2 cm) (Fig. 2).

δ15N values of soil samples were lower at the forest sites (FOREST1 and FOREST2) than at the arable/abandoned field sites (ARABLE1, ABANDONED1, and ABANDONED2) (Fig. 2), showing potential influence of synthetic fertilizers in the arable/abandoned field sites. The two forest sites (FOREST1 and FOREST2) were similar to each other and showed a depth profile of increasing δ15N values in deeper soils. δ15N values decreased slightly with soil depth at the two abandoned field sites (Fig. 2).

*3.2 Isotopic niches of earthworm species*

*3.2.1 Arable field 1*

At ARABLE1, two endogeic earthworm species were collected: *Ap. caliginosa* and *Ap. trapezoides* (Fig. 3). *Ap. caliginosa* had higher δ15N values and occupied the upper part of the isotopic space,whereas *Ap. trapezoides* had lower δ15N values and occupied the lower part of the isotopic space (Fig. 3).

Comparison of the total isotopic niches suggested niche differentiation between the two species (PERMANOVA *P* = 0.004; PERMDISP *P* = 0.38) (Appendix S1: Table S1). The core niche areas of *Ap. caliginosa* and *Ap. trapezoides* were similar in size (SEAB = 3.04‰2 and 4.18‰2, respectively) (Fig. 3, Appendix S1: Table S2, Fig. S1) and showed low degrees of mutual overlap (*ca.* 10.0%) (Figs. 3 and 4, Appendix S1: Table S3).

*3.2.2 Abandoned arable field 1*

At ABANDONED1, six earthworm species were collected, including *Ap. caliginosa*, *Ap. trapezoides*, *Al. chlorotica*, *Di. caroliniana*, *L. friendi*, and *L. rubellus* (Fig. 3). Overall, the three endogeic species, *Ap. caliginosa*, *Ap. trapezoides*, and *Al. chlorotica*, had higher δ13C and δ15N values and occupied the upper-right part of the isotopic space; the anecic species, *L. friendi*, had lower isotope values and occupied an isotopic niche slightly below those of the endogeic species; the epigeic species, *L. rubellus*, had the lowest values and occupied the lower-left part of the isotopic space; the native species, *Di. caroliniana*, showed a distinct isotopic niche position from the other species (Fig. 3).

Pairwise comparisons of the total isotopic niches further suggested niche differentiation between species of different ecological groups: *Ap. caliginosa* and *L. friendi* (PERMANOVA *P* = 0.01; PERMDISP *P* = 0.81), *Ap. caliginosa* and *L. rubellus* (PERMANOVA *P* = 0.002; PERMDISP *P* = 0.16), *Ap. trapezoides* and *L. rubellus* (PERMANOVA *P* = 0.03; PERMDISP *P* = 0.17), and *L. friendi and L. rubellus* (PERMANOVA *P* = 0.06; PERMDISP *P* = 0.12) (Appendix S1: Table S1).

Among the three endogeic species, *Al. chlorotica* had a larger core niche area (SEAb = 14.82‰2) compared with *Ap. caliginosa* (SEAb = 5.29‰2) and *Ap. trapezoides* (SEAb = 6.53‰2) (Fig. 3, Appendix S1: Table S2, Fig. S1). The percentages of overlapping SEAB between species pairs were low to moderate on average (< 50.0%), except for three endogeic and anecic species: 71.4% of the SEABof *L. friendi* overlapped with that of *Al. chlorotica*; 79.1% and 59.7% of the SEABof *Ap. trapezoides* overlapped with that of *Al. chlorotica* and *L. friendi*, respectively (Figs. 3 and 4, Appendix S1: Table S3).

*3.2.3 Abandoned arable field 2*

At ABANDONED2, the same six earthworm species as at ABANDONED1 were collected (Fig. 3). Similar to ABANDONED1, the three endogeic species, *Ap. caliginosa*, *Ap. trapezoides*, and *Al. chlorotica*, had higher δ15N values and occupied the upper part of the isotopic space, whereas the anecic species, *L. friendi*, and the epigeic species, *L. rubellus*, had lower δ15N values and occupied the lower part of the isotopic space (Fig. 3). The native species, *Di. caroliniana*, also showed a distinct isotopic niche position from the other species, indicating its unique feeding habits (Fig. 3). However, pairwise comparisons of the total isotopic niches did not show any significant niche differences between species (Appendix S1: Table S1). The core niche area was smallest in *Ap. trapezoides* (SEAb = 3.25‰2) and largest in *Ap. caliginosa* (SEAb = 11.10‰2) (Fig. 3, Appendix S1: Table S2, Fig. S1). The percentages of overlapping SEAB between species pairs were low to moderate on average (< 50.0%), except for two endogeic species: 67.5% of the SEABof *Ap. trapezoides* overlapped with that of *Ap. caliginosa* (Figs. 3 and 4, Appendix S1: Table S3).

*3.2.4 Mature forest 1 and Mature forest 2*

At FOREST1, five earthworm species were collected, including *Ap. caliginosa*, *E. lonnbergi*, *L. rubellus*, *L. terrestris*, and *O. cyaneum* (Fig. 5). As previously reported (Chang et al. 2016b), *O. cyaneum* and *E. lonnbergi* had higher δ15N values and occupied the upper part of the isotopic space, whereas the anecic species *L. terrestris* and the epigeic species *L. rubellus* had lower δ15N values and occupied the lower part of the isotopic space (Fig. 5), with *Ap. caliginosa* sitting in between. A similar pattern can be seen at FOREST2 (Fig. 5). Consistent with the results in Chang et al. (2016b), niche differentiation was confirmed in all species pairs at FOREST1 and FOREST2 except between *E. lonnbergi* and *O. cyaneum* at FOREST1 (PERMANOVA *P* = 0.99; PERMDISP *P* = 0.99) (Fig. 5, Appendix S1: Table S1, Fig. S2).

*3.2.5 Isotope discrimination between soil and earthworms across sites*

The carbon and nitrogen isotope discrimination (Δ13C and Δ15N) between the soil and earthworm species across the five study sites revealed nine potential isotopic niches of earthworms that form a continuum along the three main ecological groups, while the native species *Di. caroliniana* occupied a distinct niche position from the remaining species in the isotopic space (Fig. 6).

*3.3 Variations in niche patterns of Ap. caliginosa and Ap. trapezoides*

*Ap. caliginosa* and *Ap. trapezoides* are phylogenetically related species belonging to the *Ap. caliginosa* species complex. They were previously considered different ecomorphs of the same species, and their taxonomic status was confirmed only recently (Perez-Losada et al., 2009; Fernandez et al., 2012). The coexistence of the two species at ARABLE1, ABANDONED1, and ABANDONED2 provided us with a rare opportunity to investigate their potential niche differentiation.

At ARABLE1 and ABANDONED1, the sizes of the core isotopic niche areas (SEAB) of *Ap. caliginosa* and *Ap. trapezoides* were comparatively similar, although the core niche of *Ap. trapezoides* tended to be slightly larger than that of *Ap. caliginosa* (*Ap. caliginosa* vs. *Ap. trapezoides*; ARABLE1: SEAB = 3.04‰2 vs. 4.18‰2; ABANDONED1: SEAB = 5.29‰2 vs. 6.53‰2) (Appendix S1: Table S2, Fig. S1). The core niches of *Ap. caliginosa* and *Ap. trapezoides* were relatively separated, with the SEAB of *Ap. caliginosa* lying above that of *Ap. trapezoides* (i.e., higher δ15N values for *Ap. caliginosa* than for *Ap. trapezoides*) (Fig. 3). The percentages of mutual SEAB overlaps between the two species were only low to moderate (*Ap. caliginosa* vs. *Ap. trapezoides*; ARABLE1: 11.7% vs. 8.8%; ABANDONED1: 28.9% vs. 23.0%) (Figs. 3 and 4, Appendix S1: Table S3).

In contrast to the patterns at ARABLE1 and ABANDONED1, the core niche area of *Ap. caliginosa* was more than three timeslarger than that of *Ap. trapezoides* at ABANDONED2 (*Ap. caliginosa* vs. *Ap. trapezoides*; SEAB = 11.10‰2 vs. 3.25‰2) (Appendix S1: Table S2, Fig. S1). The core niches of the two species overlapped substantially (Fig. 3), yet the percentages of mutual SEAB overlaps were highly asymmetric (*Ap. caliginosa* vs. *Ap. trapezoides*; SEAB = 21.2% vs. 67.5%) because of the large differences in their niche areas (Figs. 3 and 4, Appendix S1: Table S3).

The total isotopic niches of the two species differed significantly at ARABLE1 (PERMANOVA *P* = 0.004, PERMDISP *P* = 0.38) but not at ABANDONED1 (PERMANOVA *P* = 0.14, PERMDISP *P* = 0.91) or ABANDONED2 (PERMANOVA *P* = 0.56, PERMDISP *P* = 0.09) (Appendix S1: Table S1).

**4. Discussion**

Our results confirmed the documented observations of isotopic niche differences between commonly recognized ecological groups of earthworms, while also further revealed deeper insights among endogeic species. Consistent with previous studies (Schmidt et al., 1997; Scheu and Falca, 2000; Pollierer et al., 2009; Melody and Schmidt, 2012; Potapov et al., 2019b,c), endogeic species had higher δ13C and δ15N values compared to epigeic species. Moreover, across the three studied land use types and all five sites, isotopic differences were also evident among endogeic species, suggesting potential niche differentiation and the need for a more refined ecological grouping system within the endogeic category.

Among the five sites, ABANDONED2 had considerably higher isotopic overlaps between species, and is the only site not showing any statistically significant interspecific isotopic niche difference. We suspected that this was due to a combination of strong C3 signals from herbaceous plants growing at ABANDONED2, C4 legacy in the soil from past land use (corn field), and great spatial heterogeneity created by patches of growing C3 (mostly forbs) and C4 (mostly graminoids) plants. Thus, our discussions below will mostly focus on the other four sites.

*4.1 Isotopic niche differentiation between endogeic species*

Both arable fields (including abandoned ones) and forests can have up to three prominent endogeic species: *Al. chlorotica*, *Ap. trapezoides*, and *Ap. caliginosa* in arable fields and *Ap. caliginosa*, *O. cyaneum*, and *E. lonnbergi* in forests. In arable fields, isotopic niche differences can be seen between *Ap. caliginosa* and *Ap. trapezoides*, with the former being more enriched in both 13C and 15N and only small to moderate mutual SEAB overlaps (8.8-28.9%) between the two species. Similarly, in forests, the three endogeic species, *Ap. caliginosa*, *E. lonnbergi*, and *O. cyaneum*, occupied distinct isotopic niches. However, in the forests, *O. cyaneum* and *E. lonnbergi* were isotopically more enriched than *Ap. caliginosa* and had no overlap with the latter, whereas in the arable field, *Ap. caliginosa* is the most isotopically enriched species with apparent overlaps with the other two species. This dramatic difference can be explained by their feeding ecology: *O. cyaneum* and *E. lonnbergi*, which are larger than the other three species, are both mesohumic endogeic (feeding on soil more enriched in 13C and 15N), while the other three species, including *Ap. caliginosa*, are all polyhumic endogeic (feeding on soil less enriched in 13C and 15N). This isotopic difference between mesohumic and polyhumic endogeic species is clear evidence distinguishing these two ecological groups.

While considered as consuming either leaf litter or soil (or both), earthworms rely on different resources for food (Curry and Schmidt, 2007; Eissfeller et al., 2013; Ferlian et al., 2014; Larsen et al., 2016; Potapov et al., 2019c, 2022). Higher δ13C and δ15N values in endogeic species compared to litter-feeding species have been attributed to feeding more on microbially processed soil organic matter, on microbial residues, or on microorganisms themselves (Potapov et al., 2019b, c, 2022). Using compound-specific stable isotope analysis (CSIA) of fatty acids, Ferlian et al. (2014) reported that fatty acid δ13C profile of *Ap. caliginosa* was closer to fungi than to bacteria, and associated this finding with the assimilation of recalcitrant carbon sources by this species. In contrast, using CSIA of amino acids to infer trophic levels, Potapov et al. (2019c) concluded that *Ap. caliginosa* relies equally on both plant material and microorganisms. So far, the endogeic species included in previous CSIA were all polyhumic (*Ap. caliginosa*, *Ap. rosea*, *Al. chlorotica*, and *O. lacteum*) (Ferlian et al., 2014; Larsen et al., 2016; Potapov et al., 2019c). However, no mesohumic endogeic species have ever been investigated. Given that mesohumic endogeic species are more enriched in 13C and 15N, we expect these species to rely more on soil microbes and microbially processed soil organic matter for food.

*Aporrectodea caliginosa* and *Ap. trapezoides* are two closely related species that, for decades, had long been believed to be ecomorphs of the same species, and were only recently confirmed to be evolutionarily distinct (Perez-Losada et al., 2009; Fernandez et al., 2012). Our SEAB analysis demonstrated niche partitioning between the two species for the first time, with relatively low isotopic niche overlaps at ABANDONED1 and ARABLE1, where the two species coexisted. Moreover, the higher δ15N values in *Ap. caliginosa* compared to those in *Ap. trapezoides* at the two sites is clear evidence that *Ap. caliginosa* consumes higher proportions of soil organic matter and microorganisms in its diet (Potapov et al., 2019c) compared to its close relative *Ap. trapezoides*.

*4.2 Generalists and specialists*

Chang et al. (2016b, 2021) proposed that under the context of ongoing invasion of jumping worms in North American forests, *M. hilgendorfi*, an epi-endogeic species, might be a stronger competitor against *L. rubellus* because the former, a “generalist”, occupied a larger isotopic niche and is capable of using a wider range of resources, which help them quickly increase in abundance and spread. In contrast, *L. rubellus*, another epi-endogeic species, has a smaller isotopic niche and is thus a “specialist” in a relative sense. It remains to be seen whether the two species will coexist in the long term (Chang et al., 2021). At ABANDONED1, we found a similar isotopic pattern among the three polyhumic endogeic species⁠—*Al. chlorotica* occupied a relatively large isotopic niche area and was thus a generalist, whereas *Ap. caliginosa* and *Ap. trapezoides* had narrower isotopic niche breadths and could both be considered as specialists. However, based on our knowledge about the three species and the earthworm fauna in the region, *Al. chlorotica* does not appear to be a strong competitor. It was never the dominant species in an earthworm community. Theory predicts that specialists perform better than generalists in their narrower niches, which may provide a potential mechanism for species coexistence (Futuyma and Moreno, 1988; Wilson and Yoshimura, 1994). Nevertheless, the coexistence of three polyhumic endogeic species at ABANDONED1 suggests that the polyhumic endogeic isotopic space may host as many as three coexisting species, including two specialists and one generalist.

*4.3 Isotopic niche space occupied by earthworms*

Taken together, species analyzed in this study demonstrated at least nine potential isotopic niches of earthworms in the two-dimensional space delimited by 13C and 15N (Fig. 6). At the lower-left of this two-dimensional space are two isotopic niches represented by the litter-feeding epi-endogeic “specialist” *L. rubellus* and the litter-feeding epi-endogeic “generalist” *M. hilgendorfi*, with the latter occupying a relatively larger niche space. Moving further towards higher δ13C and δ15N values are three polyhumic endogeic species, including a “generalist” represented by *Al. chlorotica* and two “specialists” represented by *Ap. trapezoides* and *Ap. caliginosa*. Among them, *Al. chlorotica* has the largest isotopic niche, whereas *Ap. caliginosa* is isotopically more enriched than *Ap. trapezoides*. At the upper-right of the isotopic space are two mesohumic endogeic species, *E. lonnbergi* and *O. cyaneum*. They are isotopically separated from each other and are more enriched than the polyhumic endogeic species. As for anecic species, depending on the relative amount of soil versus leaf litter they consume, their isotopic niches can be similar to either epi-endogeic species, as in the anecic species *L. terrestris*, or to polyhumic endogeic species, as in *L. friendi*. Interestingly, compared to other species, the native endogeic species *Di. caroliniana* has apparently lower δ13C values. This can indicate a stronger C3 signal that resulted from selectively feeding on forbs growing in the abandoned arable fields.

In terms of trophic position of earthworm species, the stable isotope approach highlighted an ecological diversity that is greater than generally perceived under the three simple groups—epigeic, endogeic, and anecic. Among the 10 species we analyzed, some species are isotopically distinct from each other, while others overlap almost entirely, with the only apparent difference being their isotopic niche sizes. Altogether, these species form a continuum of trophic diversity, with no clear boundary between adjacent species in many cases. This observation supports the idea that as opposed to being categorical, earthworm species form an ecological continuum with no clear boundary or gap between the commonly recognized "ecological groups" (i.e. epigeic, endogeic, anecic, etc.). This continuum, though often overlooked in modern literature, has been pointed out a long time ago by Lee (1959) and Bouché (1972, 1977), and recently by Bottinelli et al. (2020).

It is noteworthy that not all niche spaces exist at the same time in the same habitat. Some niche spaces are missing, presumably, due to the absence of the corresponding resources, as in the case of missing mesohumic endogeic species in arable fields. Additionally, the observed isotopic niches can be considered realized niches shaped by environmental factors, resources, and interspecific interactions. A species’ position in the isotopic niche space can change from one habitat to another or even within the same habitat over time, either seasonally or at different developmental stages (Schmidt, 1999; Schmidt et al., 1999; Neilson et al., 2000; Chang et al., 2016b).

It remains to be seen how epigeic species and oligohumic endogeic species fit into the framework of the 13C and 15N-delimited isotopic niches. One would expect epigeic species to be less enriched in 13C and 15N compared to epi-endogeic species, such as *L. rubellus*. However, Potapov et al. (2019c) found that *Dendrobaena octaedra* (Savigny, 1826), a commonly recognized epigeic species, relied more on bacteria and fungi for food and was more enriched in 13C and 15N compared to the epi-endogeic species *L. rubellus*. They attributed this isotopic signature to the wood-feeding behavior of *De. octaedra* at their study sites. In addition to *De. octaedra*, several epigeic species common in temperate North America and Europe, such as *Bimastos rubidus* (Savigny, 1826) and *Bimastos parvus* (Eisen, 1874), are known to be associated with decomposing wood (Csuzdi et al., 2017). In fact, a number of species live outside of the typical soil profiles, making them even more difficult to fit into the commonly used ecological categories. These species are often characterized by the microhabitat and/or environmental conditions in which they occur. While also called ecological strategies (Lee, 1985) or ecological types (Wetzel and Reynolds, 2021), terms such as corticolous (living under logs or under barks) and limicolous (living in very wet habitats) provide very little information on the ecological function of these species. There are also epigeic species living primarily under leaf litter, such as *Lumbricus castaneus* (Savigny, 1826) and *Eisenoides carolinensis* (Michaelsen, 1910). Thus, how these two different groups of epigeic earthworms fit into the current picture of isotopic niche space remains to be explored. The same can be applied to oligohumic endogeic species, which is not a common member of earthworm communities in forests and arable fields in temperate North America and Europe, and was not included in our analysis.

*4.4 Distinct isotopic niches of native North American species*

The majority of earthworm species analyzed in this study were of European origin and introduced into North America. However, two native species, *E. lonnbergi* and *Di. caroliniana*, were present in the earthworm communities we sampled. Combining isotopic labeling and feeding experiment, Chang et al. (2016b) showed that the native *E. lonnbergi* occupies a unique isotopic niche, which allows for its coexistence with both European and Asian invasive species. Our results further demonstrated that the unique feeding habits of the native species *Di. caroliniana* may allow for its coexistence with invasive European species in human-disturbed habitats, such as arable fields. Isotopic niches of native earthworm species, especially in relation to ongoing earthworm invasions in many habitats globally, is a major knowledge gap, yet this type of information is crucial in our ability to predict the outcome of interaction between native and non-native species.

*4.5 Ecological groups of earthworms revisited*

The isotopic results provided clear evidence why we opted to use Perel and Lavelle’s ecological categorization (Perel, 1975; Lavelle, 1981) when describing our data: it is relatively straightforward and easy to comprehend when applied to the species we analyzed. For instance, *Al. chlorotica* is a small endogeic species living in surface soil, whereas *O. cyaneum* is a slow-moving, medium-sized endogeic species living a little deeper in the soil. The two species have distinct isotopic signatures. In Perel and Lavelle’s system, *O. cyaneum* is a mesohumic endogeic species and *Al. chlorotica* is a polyhumic endogeic species. However, in Bouché’s system, the two ecologically distinct species belong to the same group, called “intermediate” (Bottinelli et al., 2020). This group name carries little ecological information. Similarly, *Ap. caliginosa*, *Aporrectodea rosea* (Savigny, 1826), and *Octolasion lacteum* (Örley, 1881) are all light-colored, small-bodied earthworms that can be found living in shallow soils in temperate deciduous forests in North America and Europe. In Bouché’s system, *Ap. caliginosa* and *Ap. rosea* are endogeic. However, *O. lacteum* is endo-anecic (Bottinelli et al., 2020), a confusing terminology that, for most earthworm ecologists, implies large body size and building vertical burrows deep in the soil.

Regardless of whether Bouché’s or Perel and Lavelle’s categorization is used, there are ample examples in which a species does not fit well into any categories. For example, *Octodrilus transpadanus* (Rosa, 1884), a widely distributed trans-Aegean species with medium to large body size and dark gray pigmentation, is usually considered an endogeic species (Csuzdi and Zicsi, 2003; Domínguez et al., 2015). This species can be found in meadows, riparian forests, and deciduous forests. In deciduous forests, it builds litter middens typical for anecic species. Also, its cast deposition pattern is very similar to that of the anecic species *Dendrobaena depressa* (Rosa, 1893) (Zicsi et al., 2011). In Central Europe, especially in the Apuseni Mts. (Romania), there are several similar *Octodrilus* species that lack the typical purple-red pigmentation of an anecic species but feed on leaf litter and deposit a large amount of excrement on the soil surface (Pop and Postolache, 1987). Another good example is *Allolobophora robusta* Rosa, 1895, a large-bodied Balkanic species regarded as endogeic. However, it is dark-gray pigmented and usually lives in deciduous forests where it builds permanent burrows and defecates on the soil surface, similarly to the large, anecic *Scherotheca* species of France (Domínguez et al., 2015). These examples demonstrate that there are various limitations of the traditional categorization, and highlight that a refined version of the original two systems is needed to accommodate more earthworm species.

*4.6 Stable isotopic signatures as quantitative functional traits*

The stable isotope approach has strengths as well as limitations. One obvious strength is that it is quantitative, and uses a standard procedure for collecting data and analyzing them. This should allow for comparisons across different sites and habitats, as well as facilitate the accumulation of data and meta-analyses. A major caveat is that it requires a reference material for standardization. This material is usually leaf litter, the presumed basal food resource type in a given habitat. However, seasonal variations in detritus input can make collecting representative leaf litter samples a tricky task, as is the case in our study. Additionally, the use of leaf litter as reference material ignores legacies from past land use and variations of belowground carbon input from roots in different types of vegetation.

With only a few exceptions (Uchida et al., 2004; Hyodo et al., 2008, 2012), isotope analyses of earthworms have only been applied to a handful of common peregrine species, mostly Lumbricidae of European origin. It remains to be seen if native species from around the world, such as pheretimoids in East and Southeast Asia, Glossoscolecidae and Rhinodrilidae in Central and South Americas, Eudrilidae in tropical Africa, and Acanthodrilidae and Megascolecidae in New Zealand and Australia, will show patterns consistent with those we observed in non-native species in North America.

Our study echoes calls for a trait-based approach to revisiting earthworm ecological groups (Bottinelli et al., 2020). We further propose that an isotopic database or including stable isotope data in a trait database is crucial for our fundamental understanding on species-specific earthworm ecology and for revising earthworm ecological groups. Stable isotope signatures can be considered as biochemical or trophic traits (i.e., estimators of diet) (Potapov et al., 2020). They are influenced by both environmental factors and biotic interactions, vary intraspecifically, and thus can serve as a proxy to understand the flexibility within a species. We agree that earthworm ecological groups are not functional groups (Bottinelli and Capowiez, 2021), but we need ecological categories that also help us understand the associated ecological processes and establish the mechanistic links between earthworm community structure and ecosystem function. The stable isotopes of carbon and nitrogen integrate earthworm feeding and burrowing behaviors and the temporal and spatial signals within a timeframe of several weeks or even months. These signals not only can serve as a proxy for food resource use of earthworms, but also are inherently related to the carbon and nitrogen biogeochemistry in the soil, and thus may provide insights into how earthworms affect soil C and N cycles. In addition to C and N stable isotopes, other functional traits (morphological, behavioral, physiological, and biochemical) can be added into this framework to turn the two-dimensional isotopic niche space into a multidimensional niche space (Potapov et al., 2021).

**5. Conclusions**

Using stable isotopes of carbon and nitrogen, we demonstrated that earthworms commonly found in arable fields and temperate deciduous forests in North America occupied nine isotopic niches, ranging from species feeding primarily on leaf litter to species feeding on microbially-processed soil organic matter. Although the observed isotopic patterns are, in general, consistent with the three commonly used ecological categories (epigeic, endogeic, and anecic) and with Perel and Lavelle’s more refined system, the species, altogether, form an isotopic niche continuum with considerable overlaps between species of different ecological groups, especially between trophic specialists and generalists. Additionally, along this across-group isotopic niche continuum, species within the same ecological group can exhibit considerable isotopic niche differentiation and resource partitioning. We propose that C and N stable isotopes should be incorporated as functional traits when studying earthworm ecological groups. It remains to be seen if the nine isotopic niches identified herein will be recovered when the same approach is applied to native earthworms in different taxonomic groups and biogeographical regions, and how well the outcomes can be generalized, but we are optimistic.

**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.

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