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CASE STUDY

^{14}C age of recent natural earthworm biospheroids—implications for paleosol dating

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Abstract

Earthworm biospheroids are a useful alternative to radiocarbon (^{14}C) soil dating. In this study, we undertook a series of measurements to test the ^{14}C dating potential/performance of recent earthworm biospheroid granules. A novel sample preparation protocol for ^{14}C in biospheroids was developed and elaborated at Atomki (Institute for Nuclear Research) and tested on IAEA reference materials. 24 natural biospheroid samples were extracted from five different location/environment-eight topsoils (A-horizon soils). Bomb-peak-based, high-resolution ^{14}C dating show very uniform ^{14}C results at 105.6 ± 2.6 pMC (1σ) and none of the biospheroids are older than 30 yr. It also shows that no biospheroid with a ^{14}C bomb-peak as high as that observed in the 1960s and 1990s were observed. The results confirmed that earthworms do indeed consume almost exclusively recent biogenic carbon, not other organic compounds or inorganic carbonates previously bound in the soil. The calendar age of their biospheroids were extremely close to the real (zero) age of the surface. Thus, no "reservoir effect" is seen for these macrofossils. We conclude that a biospheroid-based ^{14}C age determination method may be suitable to measure the burial time as long as earthworm biospheroids can be found in the soil.

1. Introduction

The term "soil age" is used diversely both in manner and meaning by various fields of science (archaeology, soil science, palaeoclimatology) (Tóth et al. 2018; Zacháry et al. 2020). As a complex system, soil is typically composed of inhomogeneous non-coeval organic materials making the interpretation of its age complicated (Molnár and Svingor 2010; Molnár et al. 2006; Scharpenseel and Becker-Heidmann 1992; Tóth et al. 2018; Wang et al. 1996). Various materials are potentially available to determine the age of soil samples using radiocarbon-dating (total organic carbon, carbon content of specific fractions only, as well as macrofossils, such as charcoal, wood/vegetal detritus, snails and shells) (Chichagova and Cherkinsky 1993; Hemingway et al. 2019; Scharpenseel and Becker-Heidmann 1992; Tamm and Östlund 1960; Trumbore 2000; Wang et al. 1996). However, in some cases the examined soil layer lacks a sufficient amount of radiocarbon-datable material. The age of the macrofossils may differ from the age of the surrounding sediments depending on their deposition mode, on sedimentation processes and on post-burial/deposition taphonomical processes.

The reservoir effect must also be considered during the dating of total or fractional carbon content, as the integration time of this carbon pool can be as long as several hundred years (Buró et al. 2019; Molnár and Svingor 2010; Novák et al. 2018; Scharpenseel and Becker-Heidmann 1992; Tóth et al. 2018; Wang et al. 1996).

The radiocarbon dating method can be satisfactorily used to ascertain the age of short-lived materials. However, not every sedimentary and soil layer contains well-preserved, good-quality macrophytes with such properties in sufficient amounts. The use of radiocarbon dating of secondary carbonates (specifically the earthworm biospheroids) can provide an alternative way to determine the burial age of these layers (Canti 2009), especially in the case of long-lasting soil formation with a very low sedimentation rate. Therefore contexts, but also expectations, are thus different for Late Glacial–Holocene soils or soil complexes and for Last Glacial pedogenetic horizons. For the first case, single biospheroid based dating may thus be preferable to highlight the chronological heterogeneity of the dated material.

Earthworm biospheroids are secondary carbonates whose formation and distribution within the soil/sediment complex might be driven by the vertical, horizontal, or *in situ* relocation of primary sediments (Barta 2011; Becze-Deák et al. 1997; Gerei et al. 1995; Pécsi 1990). These biospheroids are crystallized in earthworm Morren glands and excreted in soils with high carbonate content (Darwin 1881; Robertson 1936). Many species belonging to the Lumbricidae family (earthworms) can produce and excrete carbonate biospheroids in various amounts (Canti and Piearce 2003).

According to the literature, earthworms typically feed on recent biological carbon, from which they excrete the $CaCO_3$ contained in biospheroids. There are multiple reasons for this excretion: 1) to protect the organism from calcium-poisoning, 2) to moderate the pH of their body fluids against the high CO_2 content of their environment created by the precipitation of $CaCO_3$, and 3) due to the CO_2-regulation of their respiratory system (Becze-Deák et al. 1997; Darwin 1881; Piearce 1972; Robertson 1936). Radiocarbon examination of these biospheroids is only possible with an accelerator mass spectrometer (AMS) due to their small mass (<2 mg each).

Biospheroids consist of converging mostly clear rhombohedral calcite crystals which are produced by the earthworms by crystallizing micrometre-sized amorphous calcium carbonate (ACC) (Gago-Duport et al. 2008; Morgan 1981; Piearce 1972; Robertson 1936). The granules are usually ellipsoidal with an average diameter and mass of 0.4–2 mm and 1.5–2 mg, respectively. This can vary though as the natural population is a composition of younger and older singles hence the size of the granules is diverse as well (Barta 2011; Canti and Piearce 2003). The *Lumbricus terrestris L.* and *Lumbricus rubellus Hoffmeister* have one of the highest biospheroid production rates, which is estimated to be about 2.2 mg/day/single (Canti 2007, 2017; Canti and Piearce 2003; Lambkin et al. 2011). Although they typically have irregular morphology based on form and size, both in the *Lumbricidae family* and among specific species, a few characteristic shapes can be observed. Admittedly, these characteristic shapes can only be loosely connected to specific groups of species, as e.g. *Lumbricus terrestris* biospheroids are often ellipsoidal while *Lumbricus rubellus* biospheroids are often a bit thinner in comparison, the forms are not exclusive to these groups and thus can only be used as a guideline (Canti 2007).

Beside the ages, the preservation state and surface condition of the biospheroids can give information about the characteristics of the formation, the sedimentary composition of the soil, and the possible events of deposition and redeposition. As a result of these, the granules may break, their outer crystal layers may erode, or their surrounding organic coating may disappear. The presence or absence of these characteristics can indicate whether or not they were redeposited (Barta 2011).

It is important to mention that the biospheroids have been used for paleoenvironmental reconstructions using stable isotopes (Prud'homme et al. 2016, 2018; Versteegh et al. 2013) or counting (Prud'homme et al. 2015, 2019). Also, there are few radiocarbon dating attempts of Holocene soil horizons (Canti et al. 2015) and Last Glacial pedogenetic horizons in loessic contexts (Fischer et al. 2020; Moine et al. 2017).

Some publications have already reported successful applications of earthworm biospheroids to determine the burial time of soil samples (Canti et al. 2015; Moine et al. 2017; Prud'homme et al. 2016, 2018; Versteegh et al. 2013), although the number of such papers is still low. Earthworm biospheroids could even be used for paleoenvironmental reconstruction, the correlation of various pedogenetic horizons/paleosols in Last Glacial loess-palaeosol sequences, as well as the burial time of soils can be ascertained (Canti 1998, 2009, 2015; Prud'homme et al. 2016, 2018). According to these studies,

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biospheroids can be a perspective and useful alternative to classical radiocarbon dating with the highly developed AMS technology, capable of reliably measuring masses of carbon as small as 0.1–1 mg (Moine et al. 2017).

In this study, we undertook a series of measurements at the Institute for Nuclear Research, in Debrecen, Hungary to test the ^{14}C dating of recent earthworm biospheroid granules. The method was validated on IAEA reference materials (C1 [marble] and C2 [travertine]). Next, we inspected several natural biospheroid samples collected from recent (zero age) soils of various environments to check if this material yields reasonable ages for recent soils. The main goal of our study was to test the performance of biospheroid-based soil age determination on real, natural modern samples (after 1960 AD), which contain bomb-peak radiocarbon. This approach gave the unique possibility of very high age resolution dating, thus even rather small effects (5–10-yr age shift) could be revealed. On the other hand, we investigated natural soil, where the natural environmental condition was present, which could have any alternation effect on the sample age. Using modern topsoil, we know exactly the "age" of the soil (zero age, recent), no other independent age determination was needed.

At the same time, we investigated the statement that earthworms typically feed on recent biological carbon only, leaving behind the other higher apparent-age carbonaceous components of the soil, which would otherwise cause a reservoir effect (Canti 2007, 2009). Instead of the preparation procedures described in the previous literature (Canti et al 2015; Moine et al. 2017), we developed a mild acid (HCl) procedure to dissolve the outer surface (at least 10% weight loss) of the compact granules. Based on the results, we draw conclusions on the newly developed sample preparation, measurement and data evaluation methods in connection with the dating of old, buried soils.

2. Materials and methods

2.1 Study area

The applicability of the method was applied to recent surface soil samples from five different sample areas, both on multiple ($n > 1$) (18 samples) and single (6 pieces) biospheroids from the Hajdúság area (Hungary) on the Great Hungarian Plain.

The Hajdúság (1500 km^2) is located in the north, northeast part of the Great Hungarian Plain extending in a north–south direction (Figure 1). In this area fluvial (abandoned paleo channel, natural levee), aeolian deflation (deflation depression, deflation hollows) and accumulation (sand hummock) landforms can be found. Furthermore, there are several kurgans (Borsy 1969; Lóki 2014).

This area is characterized by different types of Chernozem, that were formed on the loess parent material. The main texture types of the soils are very different: sandy loam, loam, clay loam and clay. The organic carbon content of these soils varies between 3–4%. The topsoils usually contain small amounts of carbonates (3%), as they migrate and accumulate downward (>10%) in the lower part of the soil profile due to the leaching (Borsy 1969; Lóki et al. 2014; Mester et al. 2017; Novák et al. 2020).

Due to the good fertility of the soil, the vast majority of the area is under large-scale arable cultivation (Balogh et al. 2019), with cultivation of different crops. Furthermore, lower lying, lower productivity patches are used as grazing land and there is several small extent forest patches or shelterbelts (mainly *Robinia pseudoacacia*).

The study area is located in the temperate zone. In this area, the climate is warm summer and humid continental (Kottek et al. 2006). The mean annual temperature is 9.7–10.1°C, January and July monthly mean temperatures are −3°C and 21–21.5°C respectively, the average annual rainfall is 520–560 mm (Borsy 1969).

The goal of our approach was to exclude any artificial effect of some "rearing" experiments. We wanted to investigate if reworking or other effect is present in the natural environment, or not significant. We have chosen several different environments and multiple sampling depths to see if the method works properly for them, or if we observe significant "reworking" and other effect. Our results shows that the

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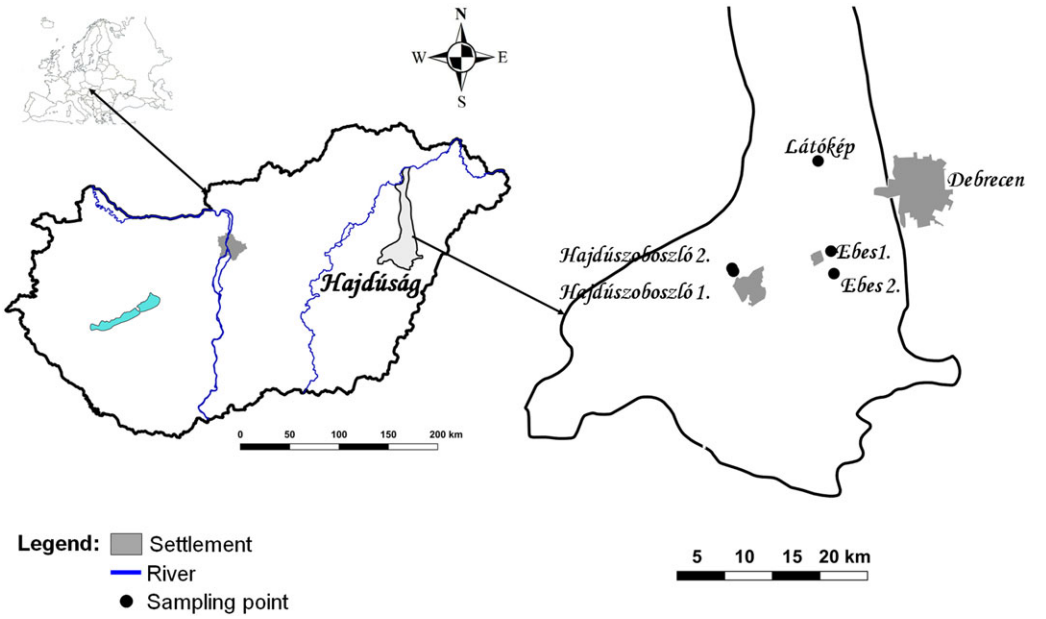

Figure 1. *Locations of the sampling sites.*

method works very well for modern, natural biospheroids, especially for geochronological purposes. All the sampled biospheroids gave realistic, young age (1995–2015 AD) within ±10 yr scatter.

2.2 Sampling locations and methods

In the Hajdúság sampling area, 5 sites with a high calcareous (Chernozem) soil layers have been identified (*E1, 2*: Ebes 1 and 2, *H1, 2*: Hajdúszoboszló 1 and 2; and *L:* Látókép), which provided a suitable environment for the formation and preservation of earthworm biospheroids.

For recent biospheroid radiocarbon measurements, a total of 8 sediment samples were collected from the 5 sites (Figure 1) at depth between 0 and 70 cm, within recent A soil horizons. Samples were collected from undisturbed environments (grazing area and forest patch/shelterbelt), meaning no significant agricultural activity had occurred there (Table 1). Three sites (Ebes 1, Hajdúszoboszló 1 and Látókép) were totally undisturbed (non-disturbed by human activity), at two other sites grazing might be present (Ebes 2 and Hajdúszoboszló 2). The very top layer (0.0–0.1 m) of the recent soil was always sampled and, in some cases, we took a second sample, from a slightly deeper horizon, but still in the A-horizon (typically between 0.1–0.2 m depth). In some access animal burrows were observed (Table 2), which were more traces of microfauna (insects, earthworms). No trace of the life of larger organisms was detected. The sediment samples were stored in a cool and dry container until preparation process began.

2.3 Separation of biospheroids from the soil samples

For the radiocarbon measurement, the soil samples (avg.: 2.5 kg each) were first wet-sieved into two separate fractions (0.56–1 mm and >1 mm). Subsequently, we used a Zeiss KL 1500 LCD type stereomicroscope for careful manual separation of the earthworm biospheroids from the other remaining soil components. A sufficient amount of biospheroids were found for the ^{14}C measurements.

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Table 1. *Carbon and oxygen stable isotope results of the 23 samples. (E1: Ebes 1; E2: Ebes 2; H1: Hajdúszoboszló 1; H2: Hajdúszoboszló 2; L: Látókép)*

Sample code (depth, m)	Characteristics of sampling site	Soil pH	Fraction (mm)	$\delta^{13}C$ (‰) VPDB (±0.2)	$\delta^{18}O$ (‰) VPDB (±0.2)
E1	Residual forest, loose humus	6.1	0.56–1	–13.3	–5.9
(0.0–0.1)	level, animal burrow			–14.1	–7.0
				–14.3	–8.4
				–13.7	–5.6
				–14.8	–8.1
Average:				–14.0 ± 0.6	–7.0 ± 1.26
E1	Residual forest, loose humus	6.1	> 1	–14.4	–7.7
(0.5–0.7)	level, animal burrow		0.56–1	–15.0	–4.3
				–14.1	–4.6
				–13.1	–5.1
				–13.9	–8.5
				–14.1	–8.7
Average:				–14.1 ± 0.6	–6.5 ± 2.0
E2	Meadow, more clayey soil, no	7.4	> 1	–14.3	–8.9
(0.0–0.1)	animal burrows		0.56–1	–14.0	–9.0
Average:				–14.1 ± 0.2	–8.95 ± 0.1
H1	Sparse grass pasture, saline	6.9	0.56–1	–11.8	–6.9
(0.0–0.1)				–13.0	–5.9
				–12.7	–7.8
Average:				–12.5 ± 0.6	–6.9 ± 0.9
H1	Sparse grass pasture, saline	6.6	> 1	–13.9	–8.7
(0.1–0.2)					
Average:				–13.9 ± 0.0	–8.7 ± 0.0
H2	Forest, clayey, few animal	6.4	> 1	–14.3	–8.9
(0.0–0.1)	burrows		0.56–1	–14.3	–8.6
Average:				–14.3 ± 0.0	–8.7 ± 0.2
H2	Forest, clayey, few animal	6.3	> 1	–14.4	–7.5
(0.1–0.2)	burrows		0.56–1	–14.1	–8.1
Average:				–14.2 ± 0.2	–7.8 ± 0.4
L	Small forest next to the arable	6.7	> 1	–11.8	–7.9
(0.05–0.15)	land		0.56–1	–12.0	–8.0
Average:				–11.9 ± 0.1	–7.9 ± 0.07
All samples avg.		6.6		–13.7 ± 0.9	–7.4 ± 1.5

2.4 Radiocarbon sample preparation and measurement method

The surface of the biospheroids had a thin organic coating of soil origin and sometimes a lime layer from the groundwater. Before the biospheroid samples processing, to develop a proper removal process of the possible foreign surface contamination, we performed some tests on IAEA international reference (C1 and C2) carbonate materials (Table 2).

We left out ultrasonic cleaning for the granules (Moine et al. 2017), as during our tests most of the granules broke into very small pieces or were very often destroyed. Using our method, the granules remained as compact bodies. Then, we apply first a H_2O_2 leaching step (30% aq.) to remove the organic

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Table 2. *^{14}C results of the prepared reference samples (literature data: Rozanski 1991; Rozanski et al. 1990)*

	IAEA-C1 (pMC ±1σ)		IAEA-C2 (pMC ±1σ)	
	Acid −	Acid +	Acid −	Acid +
	0.51 ± 0.02	0.20 ± 0.04	40.83 ± 0.16	41.15 ± 0.20
	1.09 ± 0.03	0.12 ± 0.04	41.14 ± 0.17	40.86 ± 0.24
	0.57 ± 0.02	0.13 ± 0.05	40.43 ± 0.14	40.97 ± 0.20
	0.60 ± 0.02	0.21 ± 0.05	41.55 ± 0.16	40.84 ± 0.16
	0.54 ± 0.02	0.13 ± 0.04	41.37 ± 0.15	40.98 ± 0.16
Average:	0.66 ± 0.24	0.16 ± 0.04	41.0 ± 0.40	40.96 ± 0.13
Literature data:	0.00	0.00	41.14 ± 0.10	41.14 ± 0.10

contamination than the dissolution of the surface of the compact granules by 0.01 M HCl at 75°C. After these steps all kind of surface organic material contamination is also very likely removed. In this way, we avoid the use of HNO_3 acid step. We applied MilliQ ultraclean C-free water (< 1 uS conductivity, UV sterilized, reverse-osmosis based ion exchange) for granule washing. Before use, all the test tubes were washed several times with HCl and MilliQ water in an ultrasonic bath, then after drying (at 60°C), finally they were heated/baked out at 300°C (1 hr).

We aimed for at least 10–15% dissolution when multiple biospheroids were prepared together (multiple samples to gain enough material for graphite-based AMS analyses, > 0.2 mg C each), thus first leaching by 30% H_2O_2 was used and followed by 0.01 M HCl acid wash for a short period of time. In the case of single granules, only 0.001 M of HCl solution was used, to avoid their complete dissolution.

We performed the CO_2 extraction and purification from our samples at the AMS facilities of the Hertelendi Laboratory of Environmental Studies (HEKAL). The acid treatment of snails, shells, and other carbonaceous depositions occurred in a vacuum-tight, 2-finger glass flask with a special valve. We placed the sample into the vertical finger, and the phosphoric acid in the other. After evacuating the flask, the acid (100% $H_3PO_{4\ aq}$) was poured onto the sample in the other finger. The 100% H_3PO_4 was prepared using 85% H_3PO_4 (aq) solution and P_2O_5 reagent. The applied preparation chemistry tools and steps were tested by IAEA-C1 and -C2 reference material preparation and analyses, as it is described in the text. The CO_2 produced from the carbonate in the sample was introduced into our CO_2 purification system (Molnár et al. 2013a). The carbon dioxide was cryogenically separated from the water at −78°C, using a mixture of isopropyl-alcohol and dry ice. The gas pressure was measured in a known volume, to calculate the yield. Then, the purified CO_2 gas sample was transferred to a sealed tube for graphitization.

In most cases, the last step of the sample preparation is the graphite production from the CO_2 gas samples. Graphite targets were prepared by a sealed tube graphitization method at HEKAL (Rinyu et al. 2015). After transfer of the CO_2 gas and sealing of the reaction tubes, the graphitization process consists of 2 steps: 1) 3 hr at 500°C to release the hydrogen and reduce the iron powder, and 2) 5 hr at 550°C regular graphitization process.

All of the ^{14}C measurements reported below were performed using our EnvironMICADAS AMS (Molnár et al. 2013b). Measurement time and conditions were set to collect at least 200,000 net counts for every single target in case of a modern sample. The overall measurement uncertainty for a modern sample is <3‰, including normalization, background subtraction, and counting statistics. For testing carbonate sample preparation procedures, the laboratory used international reference materials with known ^{14}C activity carbonate sample IAEA-C1 (marble) and IAEA-C2 (travertine).

The $^{13}C/^{12}C$ ratio of the sample was simultaneously measured on the EnvironMICADAS, which is essential for the correction of the radiocarbon age. The maximum age of ^{14}C measured with this instrument is 50 kyr and the average measurement time of each sample is approximately half an hour

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(Molnár et al. 2013b; Stuiver and Polach 1977; Svingor 2012). The blank level for each type of samples in case of 1 mg C (carbon content) has good reproducibility of 0.3–0.5 pMC.

The raw radiocarbon ($^{14}C/^{12}C$) results are expressed in the unit of pMC (percent Modern Carbon). The pMC unit is generally used for environmental samples (Stenström et al. 2011; Stuiver and Polach 1977), 100 pMC is equal to a specific radiocarbon activity of 0.226 Bq/g carbon, which is equal with the hypothetical specific activity of atmospheric carbon of year 1950.

$$F^{14}C = A_{SN}/A_{ON} = pMC(\%)$$

where A_{SN} is the normalized specific activity of the sample, and A_{ON} is the normalised sample activity, and their ratio is named Fraction Modern ($F^{14}C$). This ratio does not depend on sampling and measurement year. We use the pMC units as the expression in % of $F^{14}C$ ratio (Stenström et al. 2011).

Single biospheroid particles were measured with the AMS gas ion source system, because their amount of carbon content was only 30–80 µg. Thanks to the gas ion source (GIS), the AMS is able to analyze samples in CO_2 forms, for masses less than 100 µg carbon content, such as carbonate, or aerosol, collagen and water samples, avoiding the graphitization step (Molnár et al. 2020). The measurement error of the method is 1% for modern samples (Molnár et al. 2013b, 2020).

Conversion of ^{14}C values measured in pMC to calendar ages was performed using Calibomb software (http://calib.org/CALIBomb/), using data from the post-1950 atmospheric ^{14}C bomb peak (Stuiver and Reimer 1993; Hua et al. 2013). Calibrated ages are reported as age ranges at the 1 sigma confidence level (68.3%).

The pH of the soil samples was measured at the Institute of Agrochemistry and Soil Science of the University of Debrecen using an Adwa AD1020 instrument.

2.5 Stable isotope analyses

The samples were studied for $\delta^{13}C$ and $\delta^{18}O$ using a Finnigan Delta XP Plus mass spectrometer (Vodila et al. 2011). A Finnigan Delta XP Plus mass spectrometer was used for measurement of $\delta^{13}C/\delta^{18}O$ values from all sample groups. The ^{13}C content of the CO_2 samples derived from the carbonate samples were measured and expressed as $\delta^{13}C/\delta^{18}O$ values relative to V-PDB (*Belemnitella americana* from the PeeDee formation), following the equation: δ (‰) = $(R_{sample}/R_{standard} - 1)$, where R is the $^{13}C/^{12}C$ ratio in the sample or in the international standard. The precision of the measurements is better than ± 0.2‰ for $\delta^{13}C/\delta^{18}O$ (Hertelendi 1990). The preparation implies the production of a highly pure amount of CO_2 gas from each sample, because the mass spectrometers can handle only CO_2 gas (for $\delta^{13}C/\delta^{18}O$ analyses) production. In most cases, the materials to be examined were not in pure CO_2 in the gas phase. Different methods were used to convert the samples to CO_2. The carbon content conversion to CO_2 was done using the sample preparation line described above for AMS ^{14}C analyses.

3. Results and discussion

3.1 Results of biospheroid of selection

The exact data (number of pieces) of the biospheroids extracted during sieving are summarized in Figure 2.

During separation, biospheroids were found in the soil after sieving and microscopic examination. On average, per kg of sieved soil, 2–16 biospheroid (10–20 mg) particles were found in the fraction larger than 1 mm, and 7–81 specimens (36 mg) from the 0.56–1 mm fraction. Some representative raw (first line) and cleaned (second line) biospheroids per sample site are shown in Figure 3 (cleaning process is described later in Section 3.3.1).

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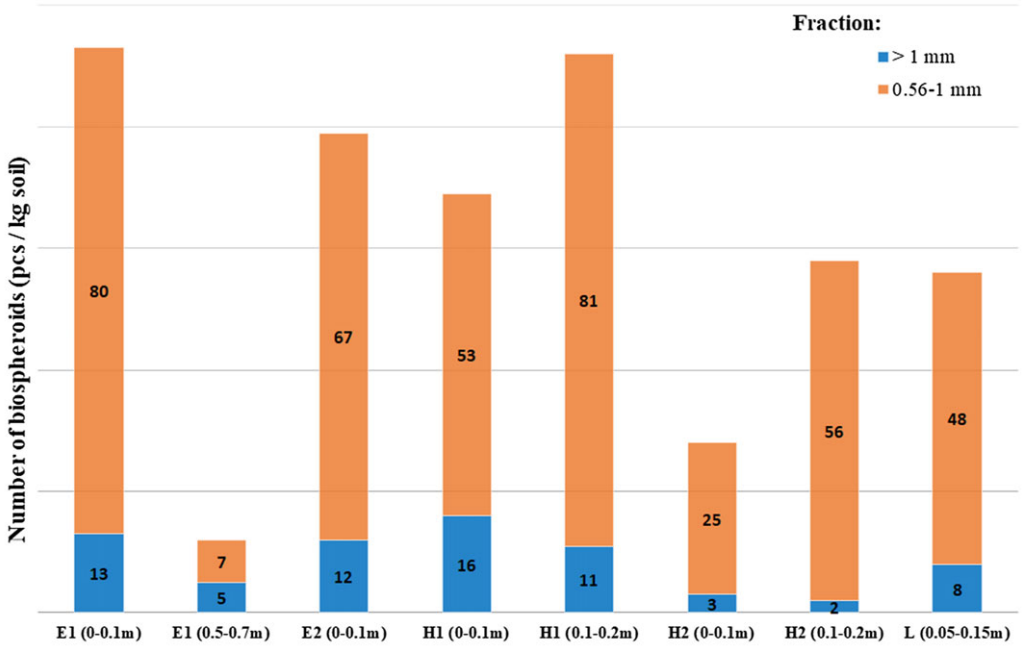

Figure 2. *Concentration of biospheroids in soil samples (E1: Ebes 1; E2: Ebes 2; H1: Hajdúszoboszló 1; H2: Hajdúszoboszló 2; L: Látókép).*

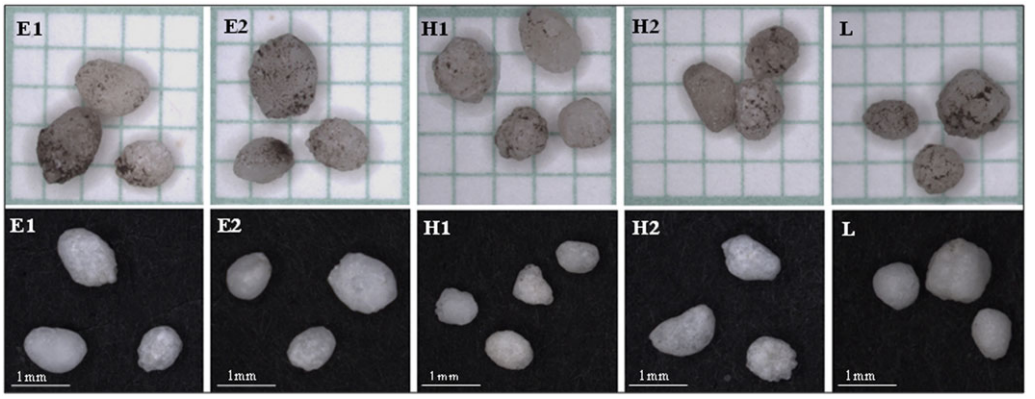

Figure 3. *Example of raw (upper line) and cleaned (lower line) biospheroid samples (> 1 mm).*

3.2 Results of stable isotopes

A total of 23 biospheroid samples from 8 different soil samples were measured for stable isotope ratios ($\delta^{13}C$ and $\delta^{18}O$) (Figure 4; Table 1).

The whole series of measurements gave an average value of $-13.7 \pm 0.9‰$ $\delta^{13}C$ and $-7.8 \pm 1.5‰$ $\delta^{18}O$ for biospheroids (Table 1). Some of the measurement results are single biospheroids, but in some cases, an average sample of some stochastically selected biospheroids was used, to obtain the minimum amount of carbon required for the multiple ^{14}C and $\delta^{13}C$ measurements. The main aim of our stable isotope analyses was simply to justify that the investigated biospheroids were not significantly different from the previously published/measured ones. In respect of ^{14}C age determination, the stable isotope composition and possible isotope fractionation is corrected/normalized in every results. That is why it is

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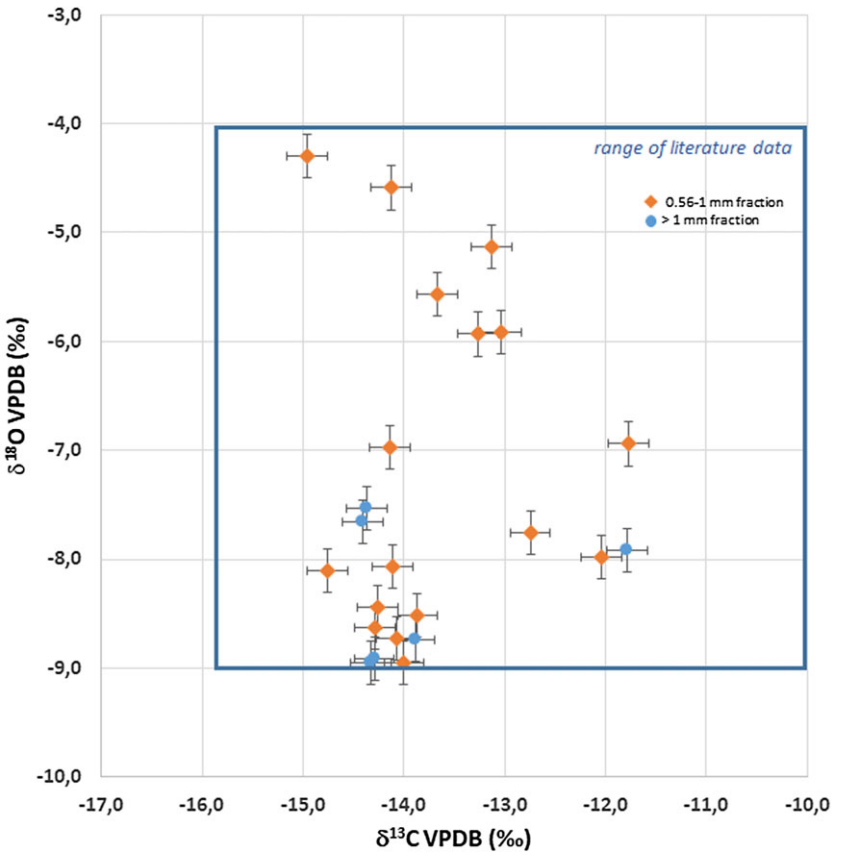

Figure 4. *Stable isotope results of biospheroid samples. Samples larger than are 1mm (blue circles) and between 0.56 to 1 mm (orange diamonds). Error margins are typical (2 σ) errors of measurement on individual sample. The blue rectangle highlights the range of literature data (Canti 2009; Prud'homme et al. 2018).*

irrelevant regarding the age result. Consequently, the scope of our study was not to present a detailed stable isotope investigation as that would need much more samples to have reliable statistics.

3.3 Radiocarbon results

3.3.1 Standard ^{14}C

The mild acid surface treatment was first performed on IAEA radiocarbon C1-C2 standard materials (20 samples). Particles from IAEA-C1 marble were the most suitable for background testing, as it has no measurable ^{14}C content, so if external impurities remain in the sample, any extra ^{14}C is observed during the measurement. First, reference materials C1 and C2, of similar mass as the samples of interest, were prepared by splitting the small reference samples into two groups of 10. We dissolved at least 10% of the sample with acid (H_2O_2 + HCl [see Section 2.4]) from the surface of one half of the sample, while the other half of the samples remained untreated for ^{14}C measurement. The purpose of the acid treatment is to remove other carbonate contaminants deposited on the surface of the sample, which could affect the actual age of the sample.

Table 2 summarizes the results of ^{14}C measurements of the IAEA-C1 and C2 standard materials (Rozanski 1991; Rozanski et al. 1990).

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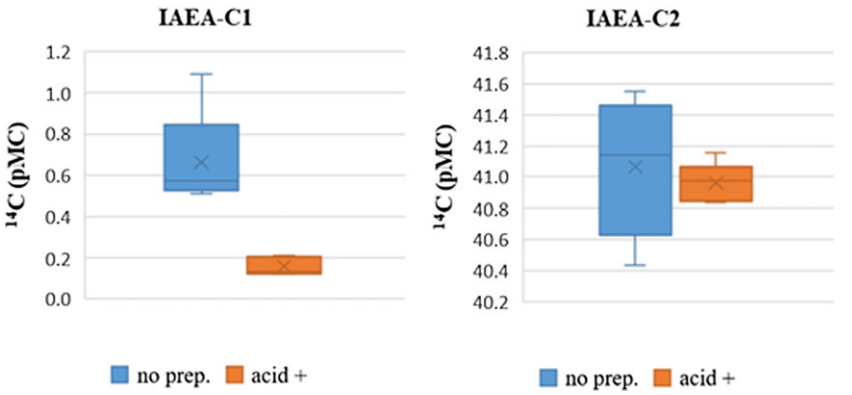

Figure 5. *Results and standard deviation of samples (before and after acid treatment).*

The measured results are shown in Figure 5. show that the acid pretreatment of the samples is appropriate and effective, because the average ^{14}C content of the prepared IAEA-C1 dropped to 0.16 ± 0.04 pMC from 0.66 ± 0.24 pMC, and the standard deviation (n = 5) is significantly smaller (0.04 instead of 0.24) when acid prep is applied. It helps significantly to get better detection limit, higher detectable maximum age. In case of IAEA-C2, after proper blank correction, the results remained equally good, but also in case of acid pretreatment the standard deviation of the repeated analyses (n = 5) is only 0.13 pMC, while it was three times higher (0.40 pMC) without the acid prep.

The international reference materials (IAEA-C1 marble, IAEA-C2 travertine) were prepared in equal amounts and co-prepared with biospheroid samples. During the preparation of the reference materials, the same tools and chemicals were used in the same amount as with our biospheroid samples. The reference graphites were measured simultaneously with the real biospheroid samples. The obtained reference results match well with the expected, literature reference values which shows the applied sample preparation and measurement methods were reliable, for IAEA-C1 and C2 materials are known.

3.3.2 Recent earthworm biospheroids

A total of 24 biospheroid (18 multiple and 6 single) samples from 8 recent surface soil samples from the study area were measured by AMS. The results are summarized in Table 3, which clearly shows a rather uniform age (within a 20–30-year interval), with averaged radiocarbon values centered on 105.6 pMC, with a standard deviation of only ± 2.6 pMC (1σ) for the multiple and single biospheroid samples.

Taking advantage of the "atomic bomb" peak of the 1960s, biospheroids from recent soils can be ^{14}C dated with great precision (calibrated age uncertainty is on the level of ± 1 year), providing a unique opportunity for an in-depth understanding of the soil mapping potential of these macrofossils.

We excluded the few-years period before the bomb-peak (possible age range is 1955–1959 AD) from the calibrated age range results (1σ) (Table 3, last column of Calendar ^{14}C age [1σ]). If there would be (any) ~60-yr-old samples (1955–1959) in the studied group of the samples (n = 24), then the 20–60-yr age range period should also be present (between 1959–1995). No samples from the period between 1959–1995 were found, only ages after 1995 are present (1σ). The very likely explanation for this observation is that all samples formed after 1995 AD, and ages from the AD 1950s can be excluded.

During 2–3 repeated measurements for each soil sample, our results reproducibly remained within a ± 3.0 pMC range (Figure 6). The ^{14}C values measured for the multiple biospheroids ranged from 103–112 pMC. The highest average (109.6 ± 3.0 pMC) radiocarbon concentration was found in the Ebes 2. (0–0.1 m) area, where the soil has a weakly alkaline pH (7.4).

One of the multiple biospheroid samples gave the lowest pMC value (Hajdúszoboszló 1. 0.1–0.2 m), which almost corresponds to ^{14}C activity level at the time of collection (100–102 pMC, for the last

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Table 3. *Measured ^{14}C ages and calculated calendar ages of the samples (avg: average, * single sample). (E1: Ebes 1; E2: Ebes 2; H1: Hajdúszoboszló 1; H2: Hajdúszoboszló 2; L: Látókép) (calibrated age results before 1960 AD are excluded, as no samples between 1960–1995 were found)*

Sample code (sample depth, m)	Soil pH	AMS lab code	Fraction (mm)	Samples (nr. pieces)	Total weight (mg)	^{14}C pMC (1σ)	Calendar ^{14}C age (1σ)
E1	6.1	DeA-13449	> 1	2	3.5	106.7 ± 0.2	2004.8–2006.7
(0.0–0.1m)		DeA-13456		3	5.3	104.9 ± 0.2	2008.2–2009.2
		DeA-13736	0.56–1	9	9.1	104.4 ± 0.3	2008.8–2009.2
		DeA-16061		1*	0.8	106.2 ± 1.1	2006.4–2011.2
		DeA-16062		1*	0.6	104.2 ± 1.0	2010.9–2014.9
		DeA-16063		1*	0.5	103.3 ± 1.1	2012.9–2018.3
Average:					3.3	104.9 ± 1.3	2006.6–2013.6
E1	6.1	DeA-13737	> 1	3	8.3	104.9 ± 0,3	2007.9–2009.2
(0.5–0.7m)		DeA-13738	0.56–1	14	6.4	104.6 ± 0.3	2008.4–2009.4
Average:					7.3	104.8 ± 0.2	2008.6–2008.8
E2	7.4	DeA-13457	> 1	2	3.7	111.9 ± 0.3	1994.7–1996.3
(0.0–0.1m)		DeA-13739		2	3.1	109.1 ± 0.3	2000.1–2001.7
		DeA-13453		3	8.9	105.6 ± 0.3	2007.4–2009.1
		DeA-13740	0.56–1	9	6.7	111.9 ± 0.4	1994.4–1996.2
Average:					5.6	109.6 ± 3.0	1999.3–2001.0
H1	6.9	DeA-13448	> 1	2	3.2	106.1 ± 0.3	2005.6–2007.8
(0.0–0.1m)		DeA-13455	0.56–1	2	4.2	105.5 ± 0.2	2007.6–2009.1
		DeA-16058		1*	0.3	100.9 ± 1.4	2012.1–2017.5
		DeA-16059		1*	0.3	106.2 ± 1.8	2000.1–2013.7
		DeA-16060		1*	0.5	102.0 ± 1.1	2007.3–2018.3
Average:					1.7	103.1 ± 2.8	2007.4–2015.6
H1	6.6	DeA-13741	> 1	4	8.6	102.6 ± 0.3	2012.1–2017.5
(0.1–0.2m)							
Average:					8.6	102.6 ± 0.3	2012.1–2017.5
H2	6.4	DeA-13742	> 1	4	8.7	106.5 ± 0.3	2004.9–2007.2
(0.0–0.1m)		DeA-13743	0.56–1	11	8.3	104.7 ± 0.3	2008.3–2009.2
Average:					8.5	105.6 ± 1.2	2005.5–2009.3
H2	6.3	DeA-13744	> 1	3	6.1	107.0 ± 0.3	2002.6–2006.9
(0.1–0.2m)		DeA-13745	0.56–1	11	7.4	104.8 ± 0.4	2008.12–2009.2
Average:					6.8	105.7 ± 1.6	2004.5–2009.9
L	6.7	DeA-13746	> 1	3	9.0	105.6 ± 0.4	2007.3–2009.2
(0.05–0.15m)		DeA-13748	0.56–1	11	9.3	104.7 ± 0.4	2008.3–2009.2
Average:					9.2	105.1 ± 0.7	2008.1–2008.9
All samples avg.:	6.6	—	—	4.3	5.0	105.6 ± 2.6	1993.7–2016.5
Multiple samples avg.:	—	—	—	5.4	6.7	106.2 ± 2.5	1990.7–2015.7
Single samples avg.:	—	—	—	1	—	103.8 ± 2.2	2007.3–2014.1

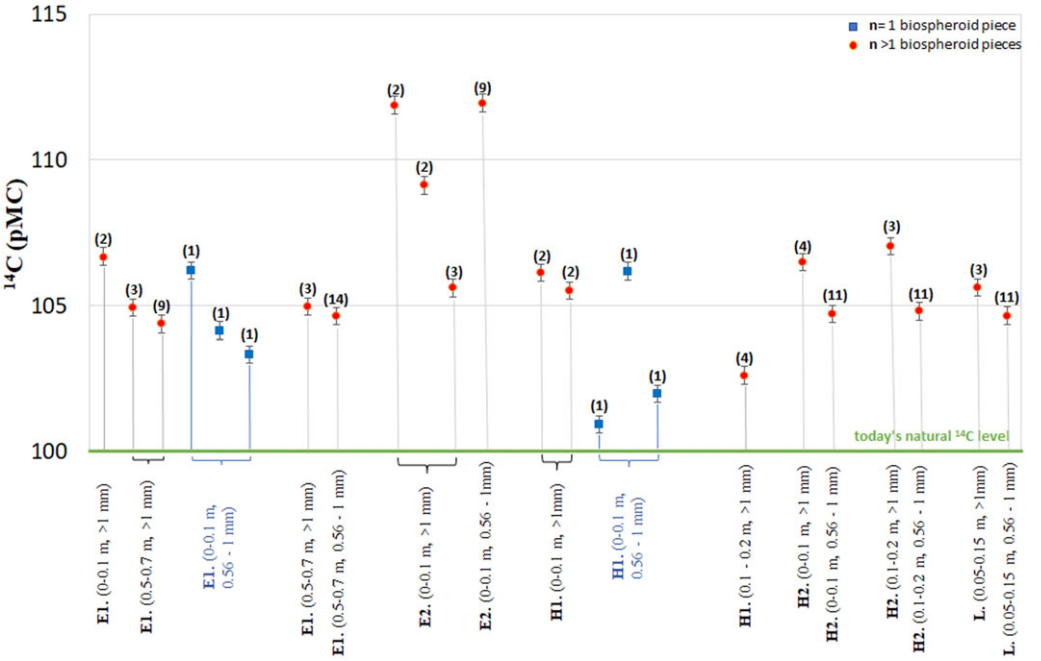

Figure 6. *Measured ^{14}C (pMC) values of earthworm biospheroid samples, indicated by piece numbers.*

10 yr). This represents a very young multiple biospheroid sample up to a few years old. We randomly selected 18 multiple and 6 single biospheroid samples from the 100 biospheroids collected from each of the 5 sites. When calibrating samples, the CaliBomb software typically gives the period before 1965 with a less than 5% probability of a possible age of origin. The single biospheroid samples came from Ebes 1. (0–0.1 m, 0.56–1 mm) and Hajdúszoboszló 1. (0–0.1 m, 0.56–1 mm), where the largest numbers of earthworm biospheroids were found during the separations.

As the measured ^{14}C values (except for one sample: Ebes 2. [0–0.1 m]) covered a rather narrow range (for 18 different multiple samples: 105.6 ± 2.6 pMC), the calculated calendar ages (Figure 7) consequently also designate a relatively narrow time interval (cal AD 1995–2015), so no age older than 30 yr was observed.

In the six single biospheroid samples, an extremely narrow time interval is shown by the calendar ages were derived from the ^{14}C measurement. This period covers the period 2005–2015, that is, 2–12 yr of age compared to the date of sampling (2017). An age interval of 8–12 yr was also obtained for two multiple biospheroid samples of the deepest sampled soil layer Ebes 1. (0.5–0.7 m). Sample Hajdúszoboszló 1. (0.1–0.2 m) combining 4 biospheroids gave an age ~ 2015, a bit younger than other multiple biospheroid samples.

In the case of multiple biospheroids, each measured sample is usually the result of 5–10 biospheroid explorations, so each measured ^{14}C based age is considered an average. The "averaged age" of 10 ± 2 yr can be said to be a very characteristic, well-reproducible average age for biospheroid samples extracted from today's topsoil, as we obtained this result in 14 of the 18 measurements. In the case of the examination of single particles, a total of 6 stochastic biospheroid samples (3 per soil sample in two sites) were measured with AMS ^{14}C using the gas ion source technique (GIS-AMS, Molnár et al. 2020), the results of which are shown in (Table 3). Consistent ^{14}C values (102–106 pMC) were obtained for single particles, and half of the samples resulted in ages younger than 5 yr compared to the sampling date (2017). The obtained ^{14}C age results from the 2 grazing sites (Ebes 2 and Hajdúszoboszló 2) were not significantly different from the three non-disturbed ones.

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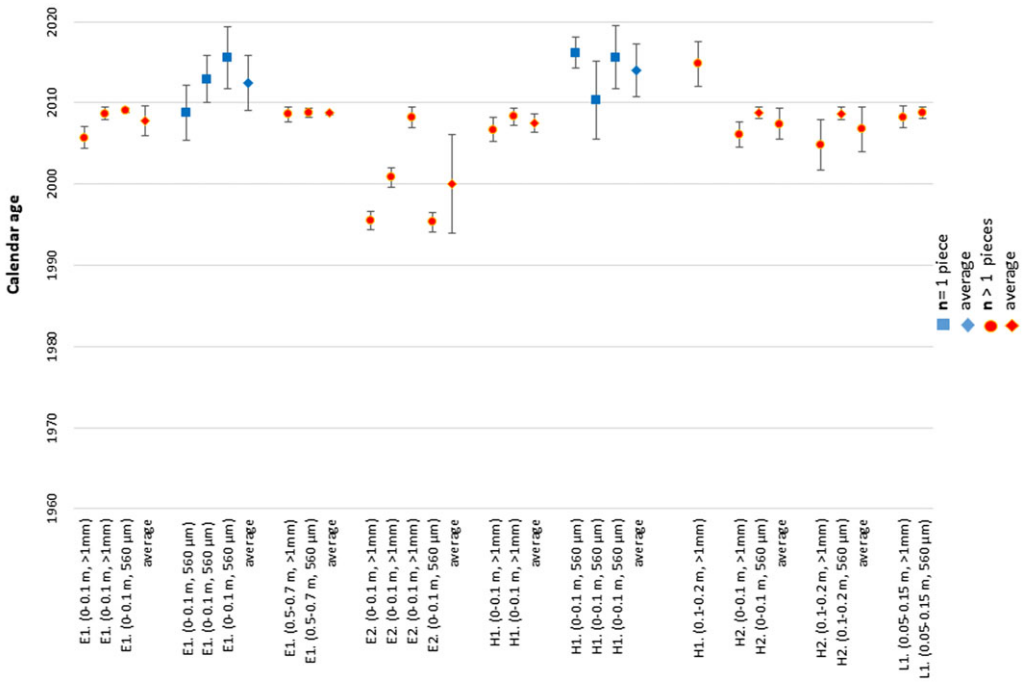

Figure 7. *The calculated calendar age of the recent biospheroid samples.*

Since the measured ^{14}C values cover a rather narrow range, the average radiocarbon concentration for the 6 different samples was 103.8 ± 2.2 pMC. Consequently, the calculated calendar ages also designate a relatively narrow time interval (2000–2017) (Figures 6–7).

4. Summary and conclusion

A new perspective research direction is the use of secondary carbonates—more specifically the application of earthworm biospheroids in measuring soil age ^{14}C. These are highly sensitive paleoenvironmental indicators, which makes them suitable for paleoenvironmental reconstruction. This fact also motivated us to start studying these calcite earthworm biospheroids. Our goal was to develop and elaborate a sample preparation protocol for earthworm biospheroids for the ^{14}C method and test it on international IAEA (C1 and C2) reference materials. Comparing it to the previously published other protocols (Canti et al. 2015; Moine et al. 2017), we did not crush the biospheroid bodies but kept them intact. We dissolved their outer surface effectively in a very controlled way, while using less aggressive chemicals. Subsequently, the applicability of the pretreatment method was checked on several biospheroid samples obtained from recent soils (from recently buried surfaces) from different environments (residual forest, meadow, grass pasture, forest). As the age of the recent topsoil (as burial time) is known, this can be considered essentially "zero age," as not buried yet. Furthermore, taking advantage of the "atomic bomb" peak of the 1960s, biospheroids from recent soils can thus be bound with very high accuracy (calibrated age uncertainty is on the level of ±1 year), thus they are very useful for this study. It is providing a unique opportunity for an in-depth understanding of the soil burial time mapping potential using these macrofossils (earthworm biospheroids).

As a result of the sieving of the soils and the selection of biospheroids, 5–10 grains (1 mm <) were found on average from 1 kg of soil samples, while 50 specimens were found between 0.56–1 mm. In the case of the radiocarbon IAEA standard materials and both the multiple and single biospheroids from

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recent, surface soils, we found that the mild acidic (10% surface solution) pretreatment was successful and expedient.

A total of 23 stable isotopes, 18 samples of ^{14}C, 6 single biospheroids and 20 standard samples were measured for recent topsoil samples collected from 5 different areas.

Based on the $\delta^{13}C$ results of the investigated 23 biospheroid samples, their carbon content is from mostly biogenic origin; the measured $\delta^{13}C$ (–13.7 ± 0.9‰ on average) and $\delta^{18}O$ (–7.4 ± 1.5‰ on average) results are in a good agreement with the published stable isotope proportions ($\delta^{13}C$: −10‰ – −15‰, and $\delta^{18}O$: −4‰ – −9‰ VPDB, respectively) (Canti 2009; Prud'homme et al. 2018).

The 24 (multiple and single) biospheroid samples extracted from, recent soils near Debrecen show extremely uniform ^{14}C results. The average radiocarbon concentration is 105.6 pMC ± 2.6 pMC (1σ). It means a rather small standard deviation (2.6 pMC) for all the biospheroid sample measurements (n = 24).

None of the 8 recent topsoil (A-horizon) samples collected in 5 different sites yielded biospheroids older than about 30 yr. It is clear from the fact, that we have not observed even a single biospheroid with such high a bomb-peak ^{14}C result as it was typical of the 1960s and 1990s. As all our results of 560 µm–1 mm sized biospheroids collected from recent topsoil show quite recent ages (10–25 yr), it suggests that the method might be suitable to measure the burial time, as long as earthworm biospheroids can be found in the soil layer. The expected overall uncertainty of this burial time analyses is rather small, within the margin of analytical error of the radiocarbon dating method, considering the typical uncertainty of the other soil dating methods. The potential and possible errors of the method should be further tested and verified on already buried soil layers, where the burial time can be measured/estimated in an independent way.

Our observations confirm that earthworms do indeed consume almost exclusively recent biogenic carbon, not other organic compounds or inorganic carbonates previously bound in the soil, and thus the age of their biospheroids is extremely close to the real age of the surface. No single biospheroids older than 30 yr were found in the studied soils. No "reservoir effect" is seen for these macrofossils. Thus, we conclude that biospheroid-based ^{14}C age determination method may be suitable to measure the burial time as long as earthworm biospheroids can be found in the soil. Is a very promising tool to estimate the age of soils, at least in similar environment as it is now in the Carpathian Basin. Further studies might be needed to test possible different effect of other type environment. The method was tested in 5 locations for soils growing in different environments. We obtained positive results in all investigated cases, i.e. the aging worked well on recent samples. There are a few examples in the literature when the results obtained were successfully compared with other (conventional) ^{14}C dating and with the OSL method (obviously, the results were not given by the bomb tip with a high-precision verification error band, but by the other methods with wider error ranges). Currently, we are not aware of any circumstances that would justify the use of the method not working in the past (Moine et al. 2017).

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