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Use of X-ray Microtomography to Detect Lead-shot Microparticles in Earthworms *Lumbricus terrestris* Linnaeus, 1758 (Clitellata: Lumbricidae)

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Abstract: A pilot study using a non-destructive imaging technique to detect the presence of microparticles of lead shot in earthworms *Lumbricus terrestris* was carried out. Earthworms play a crucial role as inhabitants of soil communities, constituting a substantial portion of their biomass. They are regarded as valuable indicators providing insights into the functional status of soils and the deposition of heavy metals. The differentiation of lead-shot microparticles from other metallic or organic particles was achieved using high-resolution X-ray computed microtomography. Applying this method allowed the reconstruction of the internal structure of the samples without compromising their integrity.

Key words: earthworms, X-ray computed microtomography, shooting range, lead shots

Introduction

Earthworms are an essential part of the soil fauna and are considered an important indicator of soil health and quality (Edwards 2004, Sizmur & Hodson 2009). They consume topsoil detritus and accumulate lead from the transformation of metallic products in shooting range areas (Darling & Thomas 2005). The bioaccumulation of lead compounds and their elevated levels can be detected in the bodies of earthworms, reaching levels indicative of toxic stress in animals consuming those (Booth et al. 2003). This is also valid for other animal species using food resources from the same biotope (Gish & Christensen 1973, Ireland 1977, Ma 1982, Vyas et al. 2000). There is also evidence to suggest that earthworms can accumulate lead by both gastrointestinal and dermal uptake (Saxe et al. 2001).

Earthworms and other soil-dwelling invertebrates that feed on lead-contaminated plants have been found to contain increased lead levels (Hui 2002, Labare et al. 2004). Lead measurements in earthworms from a trap and skeet shooting range were found to range between 660 and 840 mg/kg (Vyas et al. 2000) and these concentrations were deemed toxicologically significant.

The present study belongs to a series of ecotoxicological examinations conducted at the former *Loven Park – Sofia* shooting range where lead ammunition has accumulated (Ilieva et al. 2017). Although the shooting range is no longer operational, it has been utilised for shooting activities until the middle of the last century. Preceding toxicological tests revealed that the lead levels in the soil within the shooting range area repeatedly exceeded the maximum permissible concentrations.

Furthermore, increased lead concentrations were also measured in plants, mushrooms and earthworms.

Materials and Methods

Earthworm collection

The earthworm sampling sites were situated within the soil layers of the lead-contaminated shooting range. Earthworms were manually collected by digging and were transported to the laboratory where they were rinsed clean with water (individually, one by one) of adhering soil particles. Earthworm samples were then washed, dried and frozen at –20ºC in 100 ml plastic containers.

Analysis with X-ray fluorescence spectrometry (XRF)

Earthworms were thawed, rinsed in water, dried and analysed by X-ray fluorescence spectrometry (XRF), which was used to predict the presence of Pb. Fischerscope® X-ray diffraction spectrometer was used with a high-resolution CCD colour camera for optical monitoring of the measurement site along the primary beam axis. Data was processed using a Fischer Win FTM® software system and a Dell® computer configuration at the Laboratory of X-ray Diffraction Methods and Computed Tomography of the Institute of Physical Chemistry "Acad. Rostislaw Kaischew".

Analysis with X-ray computed microtomography

The 3D microtomography and processing were carried out at the Laboratory of X-ray Diffraction Methods and Computed Tomography of the Institute of Physical Chemistry "Acad. Rostislaw Kaischew". The study was done with an X-ray computerised microtomograph Bruker SkyScan 1272 under the following conditions: voltage of the X-ray tube 60 kV, current 166 µA, filter Al 0.25 mm and size of the 3D voxels 10 µm. Scanning consisted of seven parts with a total length of 4.4 cm and a duration of 19:26 min., or a total of 136:03 min.

Earthworms were wrapped in polyethylene film, then placed in a plastic cylinder with an internal diameter of 1 cm. Paper dividers were also placed between the individual earthworms to distinguish them. The total scanned volume was 3.45 cm^2.

Absorption of Fe, Cu, Pb and its oxides were calculated from publicly available X-ray absorption coefficient tables maintained and updated by the National Institute of Standards and Technology (NIST). The calculation methodology was described in the articles by Chantler (1995, 2000, 2005). X-ray absorption was calculated by the Beer-Lambert law:

$$T = \frac{I}{I_0} = e^{-\mu l}$$

where µ is the linear absorption coefficient, which depends on the material and the energy of the X-rays, and l is the path of the beam through the sample (in this case, it is the lead particle). The contrast positive star-shaped artefacts appear at $T<0.1$ and this limit is empirically determined; it is valid only for the apparatus and method used to reconstruct the 3D images.

Analysis with inductively coupled plasma spectrometry

Lead quantification in earthworms was performed by the methods of inductively coupled plasma spectrometry. Spectrometers Varian Vista-MPX CCD Simultaneous ICP OES, Varian Australia, and Plasma Quant MS S-NR 105000-AQ032, Analytik Jena AG, Germany, were used. The survey was conducted in the Laboratory of Ecology and Technical Tests "Aquaterratest", Sofia.

Results

The presence of lead in earthworms was detected qualitatively in predictive studies using XRF, with a spectrum showing unusually high content of Pb in the worm sample (Fig. 1). Other samples or points of measurement also showed a significant presence of Pb, though not as prevalent as in the spectra of Fig. 1. Apart from the Pb, the spectra showed also some other components of the soil: Ca 3.7 keV, Fe 6.4-6.5 keV, Cu 8.1-8.2 keV and Zn 8.6-9.7 keV. Although with small intensity, Ag peak 22.1 keV was always present. Thus, XRF analysis of earthworms collected from the firing range area revealed elevated levels of lead as well as the presence of iron and zinc. However, due to high content of organic mass (not detectable by XRF), quantitative results could not be obtained. In addition, this method could not detect whether the presence of lead in the earthworms' bodies was due to digestive or dermal absorption.

X-ray computed microtomography

The earthworms were low-absorbing to X-rays and their body parts were difficult to distinguish. However, areas with concentrated objects of different materials with different X-ray absorption were observed. These areas (Fig. 2) were arranged in "chains" that outlined the worms and represented the contents of the digestive tract.

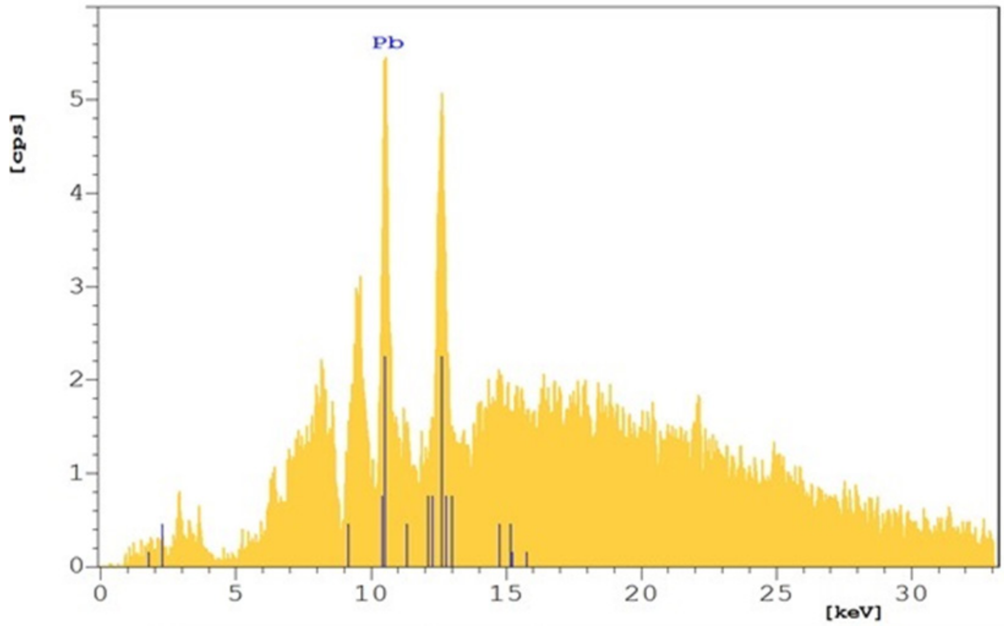

Fig. 1. XRF analysis of earthworms from a high-intensity shooting range. The presence of lead is indicated by characteristic peaks at 10.5 keV and 13 keV. Peaks indicative of Fe, Zn and Cu (not shown) were also observed.

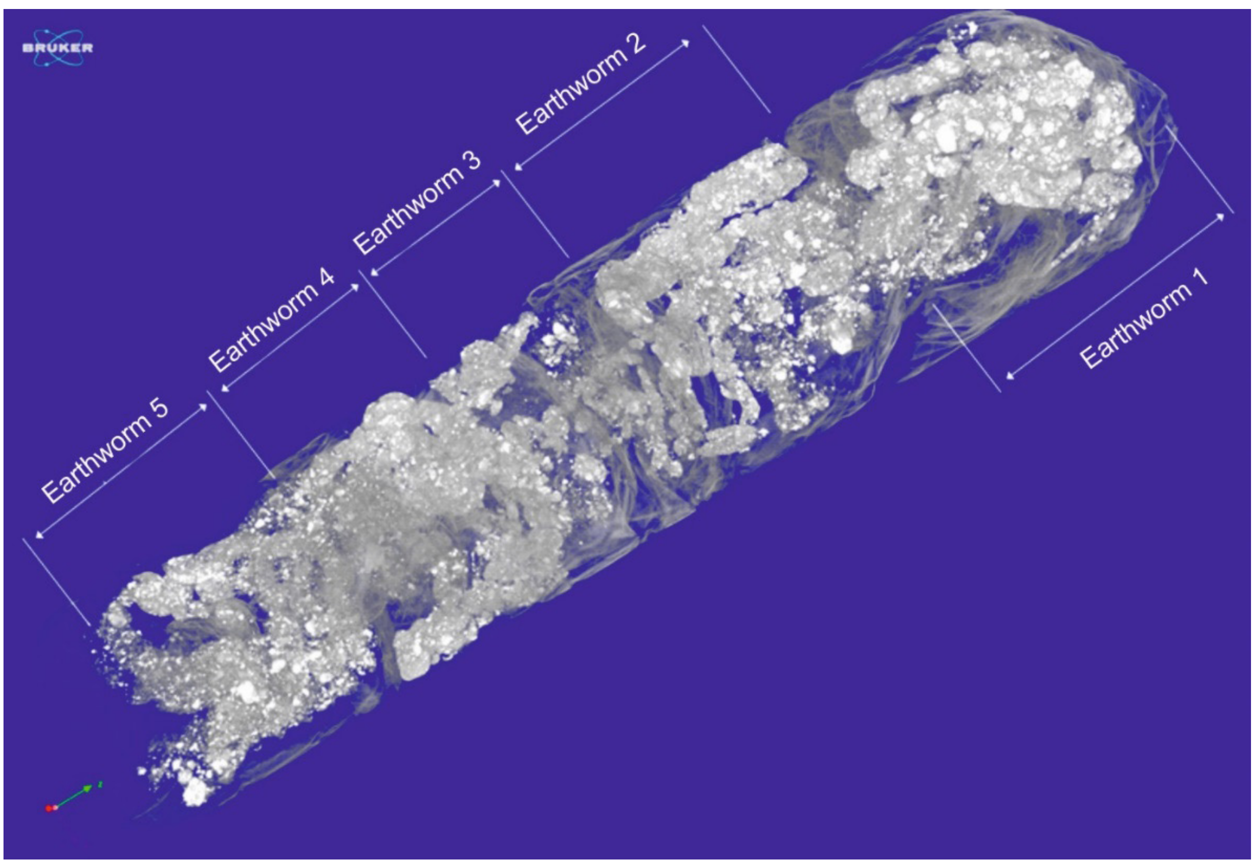

Fig. 2. Three-D volume rendering of the five earthworms in the sample. The highly absorbing parts (white colour) are situated in regions inside the earthworms. The polyethylene film with which the earthworms are wrapped is also visualized.

Figs. 3 and 4 represent typical cross sections obtained by tomographic scanning of earthworms. The grey levels corresponded to the absorption of X-rays. Lighter grey levels represented higher absorption. Strongly absorbing materials, e.g. metals, were white in colour. Sand, wood, minerals Al, Mg, etc., would be of grey colour. Thus, the recognisable grey "phases" were as follows: black – air bubble, darkest grey – worm tissues, and brighter spots – various dense particles resulting from digestive absorption. The plastic cylinder containing the worms is visible as a dark grey circle. The nylon foil used to wrap the worms is also visible. The most absorbing particles are deliberately overexposed and allow visibility of the less absorbing parts.

Generally, the bright objects are clustered mostly in circular areas suggesting that these were cross-sections of the worms' stomach tracts. Different tissues and parts of the worms were not distinguishable. The brightest objects are always surrounded by darker areas of obviously different material.

Computerised tomography could resolve materials only as different phases as defined in thermodynamics. Therefore, one might look for Pb or other heavy metals only in form of metallic Pb oxidized Pb or Pb oxide particles. Tomography could not resolve between such particles without spatial calibration of levels of grey. Anyway, particles with very low transmission, usually below 5-10%, produced specific star-like artefacts (Figs. 3 and 4). Thus, equation (1) for each material and energy sets a size above which the particle would produce low transmission artefacts. If a particle with a size above that so calculated shows no artefacts, then it is of a less absorbing, i.e. lighter material. If a particle of a certain size exhibits low transmission artefacts then it cannot be of a material that has a transmittance above approximately 0.1. The limit $T<0.1$ has been empirically established and is valid only for the apparatus and method used to reconstruct the three-dimensional image.

X-ray transmittance for particles with sizes equal to those measured by tomography showed low transmission artefacts. The calculations were made under the assumptions that the particles might be lead, iron or copper. Since the SkyScan 1272 tomograph used white radiation, two radiation energies of 20 and 40 keV were taken for the evaluation. At an operating voltage of 60 kV, the maximum X-ray intensity was about 20 keV. Considering that the lower energies were probably fully-absorbed, it was appropriate to use at least one higher energy, e.g. 40 keV, for the evaluation. From the tube X-ray spectrum given in the technical documentation of the device, the intensity ratio of the two energies was estimated as 0.625 to 0.375 for 20 and 40 keV, respectively. These numbers were used as weights to determine the weighted transmission. The weighted transmission was used in Table 1 to estimate the probability that the particles were of lead or heavier metal.

Table 1 indicates that all particles could be lead since their transmission is less than 0.1. However, the largest particles, those 320 μm in size, could also be copper, since their transmission would also be below 0.1. The probability of being lead was therefore flagged as low. The medium probability was the one where the transmission of copper particles of the given size was between 0.1 and 0.15, and the high probability was the one where the transmission of copper particles was above 0.15.

X-ray transmission calculations assuming that the particles observed with the microtomograph consisted of lead oxides, PbO, Pb_3O_4 and PbO_2, were also performed. The results revealed that PbO exhibited the highest absorption, while Pb_3O_4 displayed the lowest absorption. All of them had lower absorption than Pb, bit higher than Cu or Fe. From this, it could be inferred that the smallest particles (80–100 μm) are less likely to be from any of the oxides; however, the inference was not definite since their transmittance was just over 0.1. Larger particles could be from any of the oxides as the transmission was below 0.1.

Discussion

X-ray fluorescence analysis (XRF) analysis is a precise and cost-effective method for screening lead contamination. It is recommended for predicting the presence of Pb before employing further specific analyses (Perroy et al. 2014). In our study, the most distinct XRF peaks correspond to lead. Geological leaching of the soil likely accounts for the presence of Fe in earthworms. During these processes, iron-bearing minerals accumulate in the soil and are subsequently absorbed by earthworms. The presence of other metals such as Zn and Cu is of lesser concern. According to Spurgeon & Hopkin (1999), earthworms have the ability to regulate the levels of essential metals such as Cu and Zn and excrete them (unlike Pb).

Determining whether the presence of lead in the earthworm body is due to digestive or dermal absorption solely using XRF is challenging.

Can lead particles ingested by earthworms be visualised using microtomography? In this type of analysis of tomographic data, it is be possible to include other metals, e.g. Ni and Co. However, this would hardly change the picture as their X-ray absorption is close to that of Cu. Including Si, Al and

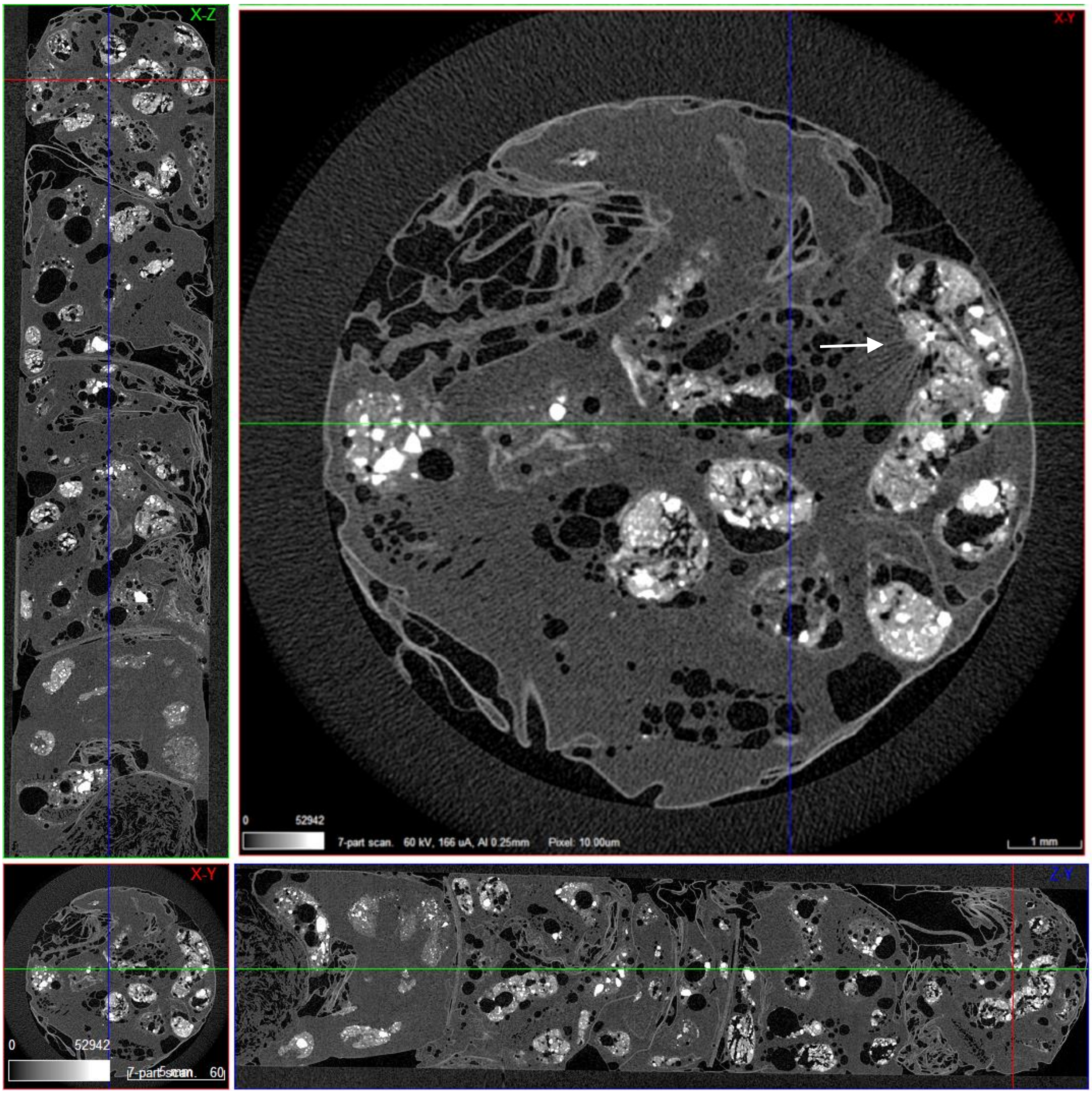

Fig. 3. A contrast positive star-shaped artefact in earthworm 1 (marked with an arrow). The three images (top-left, bottom-left and bottom-right) are three connected sections. The top-left image provides a zoomed-in view of the bottom-right image. The top-left and bottom-right images represent the entire sample in length.

their oxides is pointless as they have low absorption and are transparent at the particle dimensions measured by tomography. The inclusion of heavier metals is also uninteresting as they would be completely opaque like lead. In this sense, it should be recalled that the analysis performed does not prove that the particles are of lead and, hypothetically, they could be of any material with higher X-ray absorption than lead. Havier elements of lower abundance in soil could be excluded by common sense. However, the analysis shows that they could not be from the widespread light metals and their compounds including copper, iron, zinc, aluminium, silicon and its widespread compounds. The oxides, being the most common compounds, have lower absorption than the pure metals because oxygen is light and absorbs X-rays less. If a particle is not copper because it has a transmittance above 0.1 then it is not a copper oxide either because the transmittance of the oxide is even greater.

A more detailed study can be done by scanning the worms together with particles of the expected size of Pb or its oxides. This would allow contrast matching and distinguishing the Pb containing particles from those of Fe, Cu or Zn. As of now, we are not aware of any other comparable microtomo-

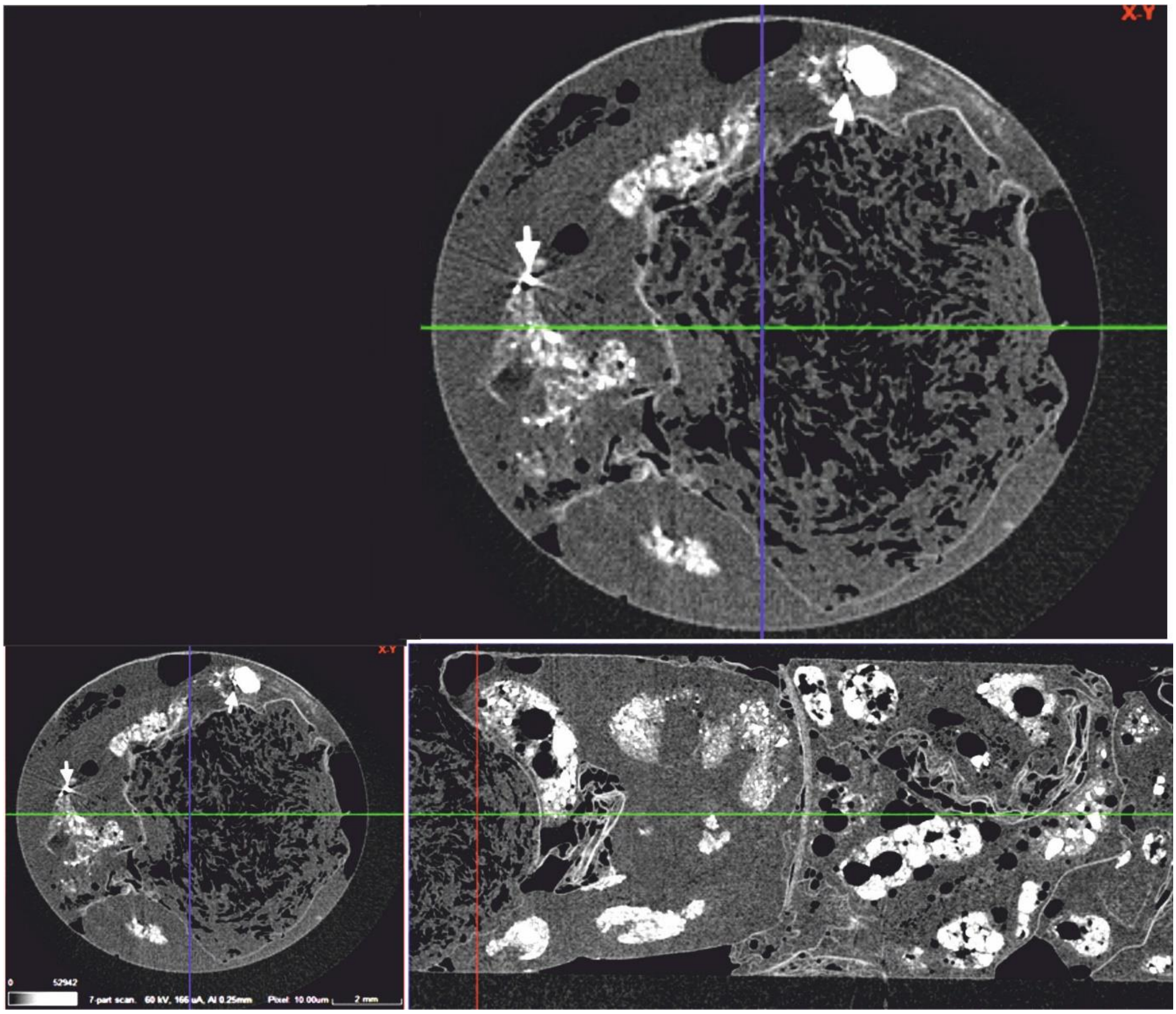

Fig. 4. Two star-shaped artefacts with positive contrast in earthworm 5 are marked with arrows. The top-left image is a zoomed of the bottom right image.

graphic study aimed at detecting lead microparticles in earthworms. Other microtomographic studies focusing on the taxonomic characteristics (Fernández et al. 2014) and the behaviour of earthworms in polluted environments (Balseiro-Romero et al. 2020) have been documented.

Analysis using inductively coupled plasma spectrometry unveiled markedly elevated lead concentrations ranging from 509.4 to 1613.9 mg/kg in earthworms, mirroring the heightened levels detected in the shooting range soils. The background sample, collected 150 m outside the firing area, displayed a significantly lower lead content of 116.07 mg/kg, corresponding to the low lead level of the soil. According to Gish & Christensen (1973), the accumulation of up to 331.4 mg/kg of lead in earthworms can prove lethal for animals that consume them.

Conclusion

Given the high levels of lead present in the ecosystem of the shooting range (in soil, plants, fungi and earthworms), the results of the microtomographic analysis confirmed the presence of lead microparticles in the digestive tract of the earthworms. High-resolution micro-computed tomography scanning of earthworms provides a non-destructive analysis that can be used to monitor lead contamination.

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Table 1. The probability that particles observed by computed microtomography are of a metal heavier than Cu and Fe and therefore could be Pb.

3D cross section	Object size µm	Weighted absorption			Probability of heavy metal (Pb) object
		Pb	Fe	Cu	
Earthworm 5					
241	140	0.03671	0.28854	0.20769	High (Fig. 3)
241	100	0.07135	0.36784	0.26983	High (Fig. 3)
321	120	0.05117	0.32309	0.23462	High
429	80	0.0997	0.42727	0.31871	High
549	160	0.02634	0.26113	0.18598	Average
839	160	0.02634	0.26113	0.18598	Average
1047	140	0.03671	0.28854	0.20769	High
1113	80	0.0997	0.42727	0.31871	High
Earthworm 4		--	--	--	
1111	80	0.0997	0.42727	0.31871	High
1197	220	0.00973	0.2041	0.13814	Average
1287	100	0.07135	0.36784	0.26983	High
1347	80	0.0997	0.42727	0.31871	High
1377	320	0.00185	0.14665	0.08693	low
1599	200	0.01356	0.22011	0.15202	Average
1753	100	0.07135	0.36784	0.26983	High
Earthworm 3			--	--	
1903	260	0.00501	0.17763	0.11457	Average
1949	160	0.02634	0.26113	0.18598	Average
2069	140	0.03671	0.28854	0.20769	High
2357	160	0.02634	0.26113	0.18598	Average
2389	80	0.0997	0.42727	0.31871	High
2447	200	0.01356	0.22011	0.15202	Average
Earthworm 2			--	--	
2853	160	0.02634	0.26113	0.18598	Average
2863	140	0.03671	0.28854	0.20769	High
2865	80	0.0997	0.42727	0.31871	High
2999	320	0.00185	0.14665	0.08693	low
3097	160	0.02634	0.26113	0.18598	Average
3309	60	0.14064	0.50796	0.39089	High
Earthworm 1			--	--	
3533	140	0.03671	0.28854	0.20769	High
3879	200	0.01356	0.22011	0.15202	Average
3931	100	0.07135	0.36784	0.26983	High
3965	200	0.01356	0.22011	0.15202	Average
4023	100	0.07135	0.36784	0.26983	High (Fig. 2)
4045	180	0.0189	0.2388	0.16777	Average
4073	100	0.07135	0.36784	0.26983	High
4191	200	0.01356	0.22011	0.15202	Average
4263	100	0.07135	0.28854	0.20769	High

References

Balseiro-Romero M., Mazurier A., Monoshyn D., Baveye P. C. & Clause J. 2020. Using X-ray microtomography to characterize the burrowing behaviour of earthworms in heterogeneously polluted soils. Pedobiology (Jena) 83: 150671. DOI: 10.1016/j.pedobi.2020.150671

Booth L., Palasz F., Darling C., Lanno R. & Wickstrom M. 2003. The effect of lead-contaminated soil from Canadian prairie skeet ranges on the neutral red retention assay and fecundity in the earthworm *Eisenia fetida*. Environmental Toxicology and Chemistry 22: 2446–2453.

Chantler C. T. 1995. Theoretical form factor, attenuation and scattering tabulation for Z=1-92 from E=1-10 eV to E=0.4-1.0 MeV. Journal of Physical and Chemical Reference Data 24: 71.

Chantler C. T. 2000. Detailed tabulation of atomic form factors, photoelectric absorption and scattering cross section, and mass attenuation coefficients in the vicinity of absorption edges in the soft x-ray (Z = 30-36, Z = 60-89, E = 0.1 keV-10 keV), addressing convergence issues of earlier work. Journal of Physical and Chemical Reference Data 29 (4): 597–1048.

Chantler C. T., Olsen K., Dragoset R. A., Chang J., Kishore A. R., Kotochigova S. A. & Zucker D. S. 2005. NIST X-ray form factor, attenuation, and scattering database (cont.): Detailed tabulation of atomic form factors, photoelectric absorption and scattering cross section, and mass attenuation coefficients for Z = 1-92 from E = 1-10 eV to E = 0.4-1.0 MeV. NIST, Physical Measurement Laboratory https://www.nist.gov/pml/x-ray-form-factor-attenuation-and-scattering-tables

Darling C. & T. Thomas V. G. 2005. Lead bioaccumulation in earthworms, *Lumbricus terrestris*, from exposure to lead compounds of differing solubility. Science of the Total Environment 346: 70–80.

Edwards C. A. 2004. The importance of earthworms as key representatives of soil fauna. In: Edwards C. A. (Ed.): Earthworm Ecology. Boca Raton, Florida, USA 2: 3–11.

Fernández R., Kvist S., Lenihan J., Giribet G. & Ziegler A. 2014. *Sine Systemate Chaos*? A versatile tool for earthworm taxonomy: non-destructive imaging of freshly fixed and museum specimens using micro-computed tomography. PLoS ONE 9 (5): e96617. DOI: 10.1371/journal.pone.0096617

Gish C. D. & Christensen R. E. 1973. Cadmium, nickel, lead, and zinc in earthworms from roadside soil. Environmental Science Technology 7: 1060–1062.

Hui C. A. 2002. Lead distribution throughout soil, flora and invertebrate at a wetland skeet range. Journal of Toxicology and Environmental Health 65: 1093–1107.

Ilieva R., Venelinov M., Stamberov P. & Ahmedova G. 2017. Lead pollution in the Shooting Area „Loven Park – Sofia". Soil Science, Agrochemistry and Ecology 51 (2): 38–43.

Ireland M. P. 1977. Lead retention in toads (*Xenopus laevis*) fed increasing levels of lead-contaminated earthworms. Environmental Pollution 12: 85–92.

Labare M. P., Butkus M. A., Riegner D., Schommer N. & Atkinson J. 2004. Evaluation of lead movement from the abiotic to biotic at a small-arms firing range. Environmental Geology 46: 750–754.

Ma W. C. 1982. The influence of soil properties and worm related factors on the concentration of heavy metals in earthworms. Pedobiologia 24: 109–119.

Perroy R. L., Belby C. S. & Mertens C. J. 2014. Mapping and modeling three-dimensional lead contamination in the wetland sediments of a former trap-shooting range, Science of the Total Environment 487: 72–81. https://doi.org/10.1016/j.scitotenv.2014.03.102

Saxe J. K., Impellitteri A. Peijneneburg W. J. G. M. & Allen H. E. 2001. Novel model describing trace metal concentrations in earthworm *Eisenia andrei*. Environmental Science and Technology 39: 7176–7814.

Sizmur T. & Hodson M. E. 2009. Do earthworms impact metal mobility and availability in soil? – A review. Environmental Pollution 157 (7): 1981–1989. http://dx.doi.org/10.1016/j.envpol.2009.02.029

Spurgeon D. J. & Hopkin S. P. 1999. Comparisons of metal accumulation and excretion kinetics in earthworms (*Eisenia fetida*) exposed to contaminated field and laboratory soils. Applied Soil Ecology 11: 227–243.

Vyas N. B., Spann J. W., Heinz G. H., Beyer W. N., Jaquette J. A. & Mengelkoch J. M 2000. Lead poisoning of passerines at a trap and skeet range. Environmental Pollution 107 (1): 159–166.

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