

Synchrotron-based micro Fourier transform infrared mapping to investigate the spatial distribution of amorphous and crystalline calcium carbonate in earthworm-secreted calcium carbonate balls

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Introduction: earthworms and calcium carbonate

Several species of earthworm, including *Lumbricus terrestris*, often called the lob worm or night crawler, secrete millimetre-scale granules of calcium carbonate (see Figure 1).¹ A number of questions are posed by these granules, not least, the function that they serve (we think they are involved in pH regulation). This article addresses questions about the mineralogical composition of the granules. The granules start off life as a milky fluid, actually a suspension of micron-scale amorphous calcium carbonate, in the rear-most of three pairs of pouches that together form the earthworm's calciferous gland. This gland is located between the 10th and 12th segments

of *L. terrestris*. The milky fluid passes from the rear-most pouch to the fore-most and, in the process, the spherulites aggregate together and largely recrystallise to calcite prior to excretion into the earthworm intestine and from there out into the soil.² The curious thing about the mineralogical composition of the soil-secreted granules is that whilst they are predominantly calcite, a common crystalline form of calcium carbonate, different polymorphs (same chemical composition but the atoms and molecules are arranged differently to give a different mineral form) of calcium carbonate can also be found in the granules—aragonite, vaterite and amorphous calcium carbonate (ACC).^{2,3} We have detected amorphous calcium carbonate in gran-

ules that are several years old, which is rather surprising. If chemists make pure amorphous calcium carbonate in the laboratory using inorganic chemistry, it is extremely unstable, transforming within minutes to the crystalline polymorphs such as calcite. There is experimental evidence that organic molecules can stabilise amorphous calcium carbonate, either by binding to the surface of amorphous calcium carbonate molecular clusters and inhibiting their dissolution and recrystallization, or by binding to Ca²⁺ ions, inhibiting or retarding their interaction with HCO₃⁻ and CO₃.²⁻⁴

In this study we wished to determine whether the ACC in the granules was stabilised by organic molecules. Aside from good old fashioned scientific curi-

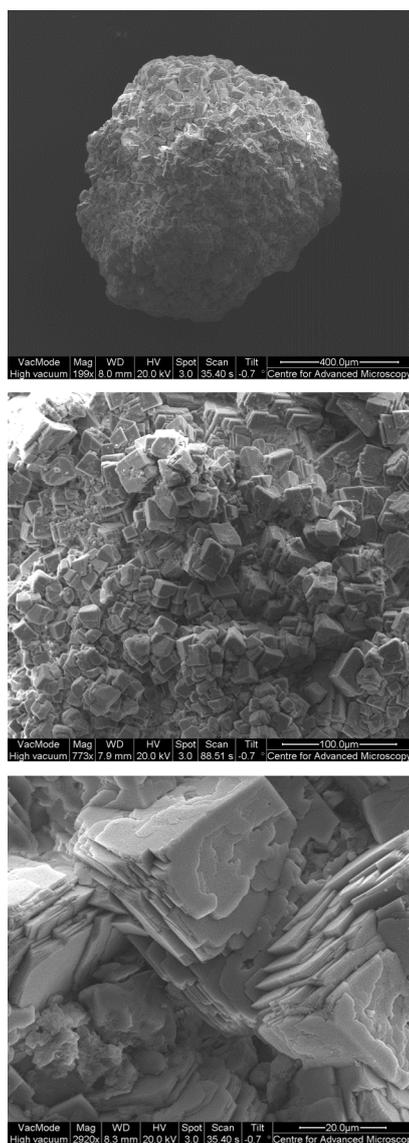


Figure 1. Granules of calcium carbonate secreted by the earthworm *L. terrestris*. Top, scale bar 400 µm; middle, scale bar 100 µm; bottom, scale bar 20 µm.

osity there are potential industrial benefits in understanding the stability of the different forms of calcium carbonate as this material is used in a whole host of industries.

Initially we worked on the bulk composition of the granules using Fourier transform infrared (FT-IR) spectroscopy but then we carried out some spatially resolved analyses with synchrotron-based μ -FT-IR mapping at Beamline B22 (Multimode InfraRed Imaging And Microspectroscopy, MIRIAM) of the Diamond Light Source, in the UK.

The bulk composition of the granules

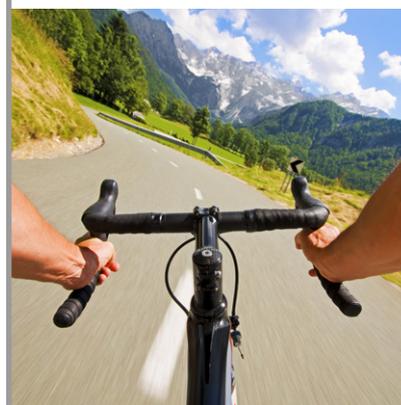
Our first challenge was to collect some granules. We purchased some *L. terrestris* earthworms from Worms Direct (Drylands, Ulting, Nr Maldon, Essex CM9 6QS, UK). Thanks to fishermen and gardeners there are good commercial supplies of this species of earthworm. We collected soils from a range of different locations around Berkshire, UK, dried the soil and then sieved it to $<250\ \mu\text{m}$. We then moistened the soil and cultivated earthworms in it for 27 days. The earthworms were removed and the soil sieved to $<500\ \mu\text{m}$. This allowed us to collect the granules, that are $\approx 2\ \text{mm}$ in diameter, which had been excreted into the soil. Subsamples of these granules were ground and the powders analysed by FT-IR spectroscopy using an A2-Technology (Danbury, USA) MicroLab portable mid-IR spectrometer fitted with a diamond internal reflection (DATR) capability in order to determine the form of calcium carbonate present and by liquid chromatography for their amino acid content.

Calcium carbonate is easily identified using FT-IR spectroscopy (see Figure 2). Crystalline carbonate phases such as calcite have distinct bands at $\sim 714\ \text{cm}^{-1}$ (ν_4), $\sim 866\ \text{cm}^{-1}$ (ν_2), $\sim 1084\ \text{cm}^{-1}$ (ν_1) and $1420\text{--}1470\ \text{cm}^{-1}$ (ν_3) whilst ACC lacks the distinct vibrational band at $\sim 714\ \text{cm}^{-1}$.

The FT-IR spectra of the bulk granules had differing peak area ratios for both the $\nu_2:\nu_4$ and the $\nu_3:\nu_4$ bands indicating differing ratios of amorphous calcium carbonate (higher ratio) to crystalline calcium carbonate (lower ratio) in the granules. We could not detect bands in the spectra corresponding to organic molecules present within the granules but this could have been due to low concentrations and detection issues.

The liquid chromatography that we carried out on the granules did indicate the presence of a range of amino acids. These may have been present initially as amino acids or proteins, our extraction method breaks proteins up into their amino acid building blocks. When we compared the amino acid content

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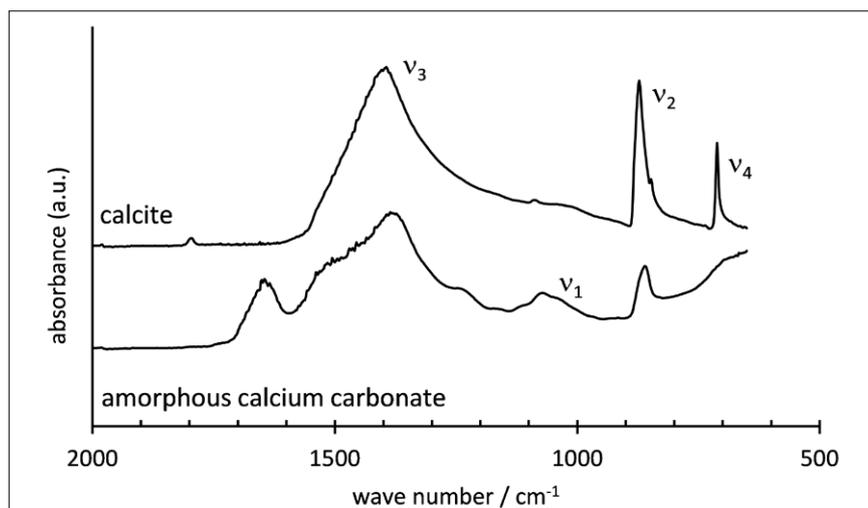


Figure 2. Typical FT-IR spectra of calcite, a crystalline calcium carbonate, and amorphous calcium carbonate.

of the granules to the peak area ratios we saw good correlations between the two for various amino acids such as aspartic acid/asparagine, glutamic acid/glutamine and phenylalanine. Although correlation does not equal causation, this suggests that the proteins

or amino acids might be stabilising the amorphous calcium carbonate. To investigate this further we decided to carry out spatial analysis to see whether the organic molecules were associated with the amorphous calcium carbonate or not.

Spatial analysis of the granules

First, we collected some more earthworm granules. We embedded these granules in EpoFIX (Struers) resin blocks and ground away the resin to expose cross sections through the granules. These samples were analysed at beamline B22 of the Diamond Light Source using a Bruker Vertex 80 V FT-IR instrument connected to a Hyperion 3000 microscope to produce μ -FT-IR maps. Spectra were collected in reflectance mode with a liquid N₂ cooled Mercury-Cadmium-Telluride (MCT) broadband detector, and at a resolution of 4 cm⁻¹ by co-adding between 128 and 1024 scans per point. Both large (*ca* 700 μ m \times 700 μ m, 20–25 μ m aperture, internal globar and synchrotron sources onto a gold mirror and zinc selenide substrate, respectively) and small (*ca* 100 μ m \times 150 μ m, 6 μ m aperture, synchrotron source, gold mirror reference) scale maps were produced of areas of the granule cross sections.

We attempted to map out the distribution of amorphous calcium carbonate

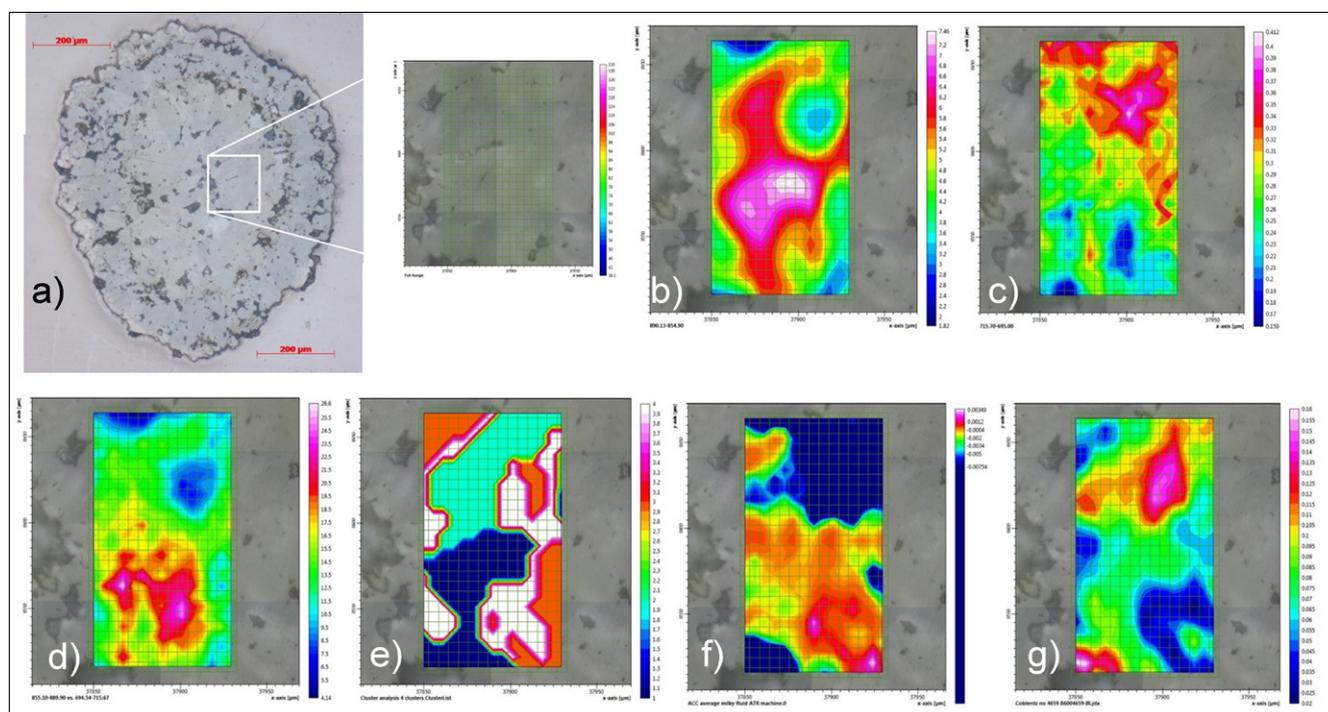


Figure 3. Images and intensity maps from synchrotron-based μ -FT-IR mapping of an earthworm-secreted calcium carbonate granule. Scale bar in a) is 200 μ m. a) Reflected light image of a cross-section through a granule; b) 855–890 cm⁻¹ (v_2), c) 695–716 cm⁻¹ (v_4) and d) $v_2:v_4$ ratio intensity maps suggesting the presence of amorphous calcium carbonate in the lower half of the map and calcite in the upper half; e) cluster analysis maps showing areas of distinctly different FT-IR spectra in the upper and lower halves of the map; f) amorphous calcium carbonate and g) calcite component regression intensity maps suggesting amorphous calcium carbonate in the lower half of the maps and calcite in the upper half. For μ -FTIR intensity maps, red indicates high intensity and blue indicates low intensity. This figure is taken from Hodson *et al.* (2015).⁵

in the granules by processing our data in four independent ways using Opus 7.2 (Bruker) software. In the most direct approach we mapped out the intensity of the FT-IR signal between 855 cm^{-1} and 890 cm^{-1} (for the ν_2 peak) and 695 cm^{-1} and 716 cm^{-1} (for the ν_4 peak) and the ratio of the two. The $\nu_2:\nu_4$ peak ratio should be highest for regions of ACC. Second, we carried out cluster analysis on the second derivative of the $855\text{--}890\text{ cm}^{-1}$ and $695\text{--}716\text{ cm}^{-1}$ regions of the spectra following vector normalisation. Although this method did not allow us to identify amorphous and crystalline calcium carbonate it identified areas with similar FT-IR spectra. Finally, we used standard amorphous calcium carbonate and calcite spectra (see Figure 2) to carry out component regression on our maps, considering the $650\text{--}1200\text{ cm}^{-1}$ range. The resulting intensity maps show how good a fit there is between the standard spectrum (either ACC or calcite) and the spectrum at each point on the FT-IR spectroscopic map. These four independent methods all identified similar areas as being likely to comprise amorphous calcium carbonate (see Figure 3). There was good correspondence between areas with a high $\nu_2:\nu_4$ peak ratio and the areas that showed the best match to the standard amorphous calcium carbonate spectrum. Similarly there was good correspondence between the areas with a low $\nu_2:\nu_4$ peak ratio and the areas that showed the best match to the standard calcite spectrum. Finally, the cluster analysis identified areas with distinct FT-IR spectral characteristics that corresponded to the high and low $\nu_2:\nu_4$ peak areas.

Having identified ACC we had hoped to identify bands related to organic compounds in our spectra as well to see whether there was any relationship between the two. We did not see a peak at 1650 cm^{-1} where the amide I band should occur and unfortunately our spectra had a broad peak in the $1570\text{--}1350\text{ cm}^{-1}$ wavenumber range where we had hoped to see distinct peaks at 1540 cm^{-1} and 1450 cm^{-1} corresponding to the amide II and lipid/amide III bands, respectively. The ν_3 peak of calcite is also present in this range (at

$1420\text{--}1470\text{ cm}^{-1}$). To date we have been unable to deconvolute this mixed component peak. We are now attempting to use another spectroscopic technique—scanning transmission X-ray microscopy—at the Diamond Light Source (Beamline I08), to do the same job using Ca X-ray absorption near edge spectroscopy (XANES) maps to identify the form of calcium carbonate present and CXANES to determine whether any of the carbon present in amorphous calcium carbonate regions is present as organic carbon rather than carbonate.

Acknowledgements

This work was funded by NERC grants NE/F009623/1 and NE/H021914/1A, a White Rose Consortium grant and beamtime at the Diamond light source under grants SM9197 and SM8989. We thank the Leverhulme Trust for financial support to the NEaer laboratory, University of York and Sheila Taylor (University of York) for provision of technical support for amino acid analyses.

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