# Carbon isotope fractionation between amorphous calcium carbonate and calcite in earthworm-produced calcium carbonate

E.A.A. Versteegh [a, *], S. Black [b], M.E. Hodson [a, c, **]

[a] University of Reading, Soil Research Centre, Department of Geography and Environmental Science, School of Archaeology, Geography and Environmental Science, Whiteknights, P.O. Box 233, Reading RG6 6DW, UK
[b] University of Reading, Department of Archaeology, School of Archaeology, Geography and Environmental Science, Whiteknights, P.O. Box 227, Reading RG6 6AB, UK
[c] University of York, Environment Department, Heslington, York YO10 5NG, UK

## ARTICLE INFO

## ABSTRACT

In this study we investigate carbon isotope fractionation during the crystallization of biogenic calcium carbonate. Several species of earthworm including *Lumbricus terrestris* secrete $CaCO_3$. Initially a milky fluid comprising micro-spherules of amorphous $CaCO_3$ (ACC) is secreted into pouches of the earthworm calciferous gland. The micro-spherules coalesce and crystalize to form millimetre scale granules, largely comprising calcite. These are secreted into the earthworm intestine and from there into the soil. *L. terrestris* were cultured for 28 days in two different soils, moistened with three different mineral waters at 10, 16 and 20 °C. The milky fluid in the calciferous glands, granules in the pouches of the calciferous glands and granules excreted into the soil were collected and analysed by FTIR spectroscopy to determine the form of $CaCO_3$ present and by IRMS to determine $\delta^{13}C$ values. The milky fluid was ACC. Granules removed from the pouches and soil were largely calcite; the granules removed from the pouches contained more residual ACC than those recovered from the soil. The $\delta^{13}C$ values of milky fluid and pouch granules became significantly more negative with increasing temperature ($p \leq 0.001$). For samples from each temperature treatment, $\delta^{13}C$ values became significantly ($p \leq 0.001$) more negative from the milky fluid to the pouch granules to the soil granules ($-13.77$, $-14.69$ and $-15.00$ respectively at 10 °C; $-14.37$, $-15.07$ and $-15.18$ respectively at 16 °C and $-14.89$, $-15.41$ and $-15.65$ respectively at 20 °C). Fractionation of C isotopes occurred as the ACC recrystallized to form calcite with the fractionation factor $\varepsilon_{calcite-ACC} = -1.20 \pm 0.52$‰. This is consistent with the crystallization involving dissolution and reprecipitation rather than a solid state rearrangement. Although C isotopic fractionation has previously been described between different species of dissolved inorganic carbon and various $CaCO_3$ polymorphs, this is the first documented evidence for C isotope fractionation between ACC and the calcite it recrystallizes to. This phenomenon may prove important for the interpretation of $CaCO_3$-based C isotope environmental proxies.

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## 1. Introduction

Many earthworm species produce calcium carbonate ($CaCO_3$) granules in specialised calciferous glands. In the earthworm

* Corresponding author. University of Reading, Soil Research Centre, Department of Geography and Environmental Science, School of Archaeology, Geography and Environmental Science, Whiteknights, P.O. Box 233, Reading, RG6 6DW, UK.
** Corresponding author. Environment Department, University of York, Wentworth Way, York, YO10 5NG, UK.
E-mail addresses: e.a.a.versteegh@gmail.com (E.A.A. Versteegh), mark.hodson@york.ac.uk (M.E. Hodson).

*Lumbricus terrestris* these occur in segments 11–12 as two pairs of swellings off the oesophagus, and one pair of pouches anterior to the glands in segment 10 (Darwin, 1881; Canti and Piearce, 2003).

$CaCO_3$ production starts by secretion of an amorphous calcium carbonate (ACC) suspension that we refer to as milky fluid. In the pouches, small spherulites (1–5 μm) in the milky fluid accrete into larger granules (≤2.5 mm). These are released into the oesophagus and excreted into the soil (Briones et al., 2008; Gago-Duport et al., 2008). The granules retrieved from the pouches and the soil are predominantly calcite, but can contain small amounts of ACC, vaterite and aragonite (Gago-Duport et al., 2008; Lee et al., 2008; Fraser et al., 2011; Brinza et al., 2013, 2014a, 2014b; Hodson et al.,

2015). The function of $CaCO_3$ production by the earthworms remains unclear but is likely related to regulation of pH and $CO_2$ concentrations in body fluids (Voigt, 1933; Aoki, 1934; Kaestner, 1967; Kühle, 1980; Versteegh et al., 2014).

It is known that considerable $\delta^{13}C$ fractionation factors exist between the different species of DIC and the various polymorphs of $CaCO_3$ (Fouke et al., 2000; Romanek et al., 1992; Szaran, 1997; Zhang et al., 1995). In addition to thermodynamics, kinetics of precipitation plays an important role in fractionation (Watson, 2004; DePaolo, 2011; Nielsen et al., 2012). Variable fractionation of carbon isotopes has been observed in different calcium carbonate biominerals suggesting that vital effects may also be relevant (e.g. Adkins et al., 2003; Auclair et al., 2003; Bernis et al., 2000; Lécuyer et al., 2012; McConnaughey, 1989; Rollion-Bard et al., 2016; Spooner et al., 2016). Despite many calcium carbonate minerals having an amorphous pre-cursor (Radha et al., 2010; Rodriguez-Blanco et al., 2011; Stephens et al., 2011) and stable ACC being increasingly observed in biominerals (Aizenberg et al., 2003; Jacob et al., 2008; Wehrmeister et al., 2011), carbon isotope fractionation between ACC and calcite has not been previously reported in the literature.

Here we present results of stable carbon isotope analyses on milky fluid collected from the calciferous pouches of earthworms, fresh granules also collected from the pouches, and older granules collected from the soil in which the earthworms were cultivated and address the question: how does granule mineralogy influence $\delta^{13}C$ values? Furthermore we make a first attempt at estimating the carbon isotopic fractionation factor between calcite and ACC, produced by earthworms.

## 2. Materials & methods

### 2.1. Experimental setup

Two soils were collected from agricultural fields in Berkshire, UK: Hamble (SU 61968 70235) and Red Hill (SU 56060 80033); both Typical Argillic Brown Earths (Avery, 1980; full soil characterisation in Table 1, Versteegh et al., 2014). The soil was air-dried and sieved to 250 μm prior to use (Lambkin et al., 2011). This ensures that no large granules are present in the soil at the beginning of the experiment and facilitates granule recovery at the end. Post-sieving soil pH and organic matter content were $7.5 \pm 0.3$ and $3.8 \pm 0.1\%$ for Hamble and $7.1 \pm 0.1$ and $7.4 \pm 0.1\%$ for Red Hill. For each replicate, 300 g of soil were mixed with one of three types of mineral water (initial $\delta^{18}O$ values $-10.0$, $-7.3$ and $-5.3$ ($\pm0.2$) ‰ VSMOW) to 65% water holding capacity (BS ISO, 1998). The moistened soil was put in a zip-lock bag with 5 g air-dried horse manure rehydrated with 10 ml demineralised water. One adult, clitellate *L. terrestris* was added to each bag. Bags were closed and kept at either 10, 16 or 20 °C. There were six replicates per treatment. Earthworms were acclimatised for three weeks, and then transferred to an identical treatment bag containing the same type and mass of soil and manure at the same temperature. Experimental details are given in Versteegh et al. (2013). After 28 days earthworms were removed from the bags, killed by dipping them in near-boiling water, and the calciferous glands were dissected out. Any $CaCO_3$ concretions present in the pouches were also retrieved, rinsed in deionised water and air-dried. Calciferous glands were put on a glass slide; MF was allowed to leak from the glands, was left to air-dry overnight, and collected by scraping it off the slide. The soil was wet-sieved to 500 μm to retrieve granules which were air-dried.

### 2.2. Stable-isotope analyses

Milky fluid and individual granule $CaCO_3$ samples were analysed

for $\delta^{13}C$ values using a Thermo Delta V Advantage IRMS with a GasBench II. The Gasbench II sample preparation device uses 100% *ortho*-phosphoric acid to transform $CaCO_3$ into $CO_2$ and hence only analyses the mineral fraction of the samples (Paul and Skrzypek, 2007). The raw $\delta^{13}C$ values were converted to the VPDB scale after normalising against NBS 18 and NBS 19 carbonate standards. The long-term standard deviation of a routinely analysed in-house $CaCO_3$ standard was <0.05‰. Statistical analysis of the $^{13}C$ data was carried out using SigmaPlot 12 for Windows 7.

### 2.3. Fourier transform infrared spectroscopy (FTIR)

Three samples each of milky fluid, granules from pouches and granules from soil were analysed by FTIR in the range $650-4000$ cm$^{-1}$ using a diamond internal reflection cell on a A2-Technology MicroLab Portable mid-IR spectrometer of the Cohen Laboratories, University of Leeds. Spectra were acquired by co-adding 512 scans with a 4 cm$^{-1}$ resolution. Crystalline carbonate phases have distinct bands at ~714 cm$^{-1}$ ($\nu_4$), ~866 cm$^{-1}$ ($\nu_2$), ~1084 cm$^{-1}$ ($\nu_1$) and 1420-1470 cm$^{-1}$ ($\nu_3$) whilst ACC lacks the distinct vibrational band at ~ 714 cm$^{-1}$ (Chester and Elderfield, 1967; Aizenberg et al., 1996; Gago-Duport et al., 2008; Rodriguez-Blanco et al., 2011). Areas for the $\nu_4$ and $\nu_3$ peaks covering the wavenumber ranges between 651 and 725 cm$^{-1}$ and 1602-1243 cm$^{-1}$ respectively were determined using the Nicolet EZ OMNIC 5.1 Software. Reference spectra for synthetic calcite and ACC were provided by Dr. Juan-Diego Rodriguez-Blanco, University of Copenhagen, Department of Chemistry.

## 3. Results

### 3.1. $\delta^{13}C$ values of $CaCO_3$

For each individual earthworm, 10 granules were analysed from the soil and one from each of the pouches (if available). For milky fluid, only one analysis per earthworm could be undertaken, but sometimes this failed because too little material was available. All analyses are reported in the Supplementary material. Three-way Analysis of Variance (ANOVA) with temperature, soil type and water type as factors indicated that there were no significant differences in $\delta^{13}C$ values of granules extracted from the soil between different treatments. In contrast, 3-way ANOVA followed by pairwise multiple comparison (Holm-Sidak method) indicated that there were significant differences in $\delta^{13}C$ values between different temperature treatments for the granules extracted from the pouches and also for the milky fluid ($p \leq 0.01$); values became increasingly negative from the 10 to 16−20 °C treatments. There were no significant differences in $\delta^{13}C$ values for either the milky fluid or granules from pouches between different soils or different mineral water treatments. Consequently, the data for different soil-water combinations but the same temperature were combined for analysis. Kruskall-Wallis One-way Analysis of Variance (ANOVA) on ranks followed by pair-wise comparison (Dunn's method) indicated that at each temperature there were significant differences between the $\delta^{13}C$ values of the milky fluid, granules from pouches and granules from soil with values becoming increasingly negative in that order (Fig. 1). Ranges of $\delta^{13}C$ values were relatively narrow for milky fluid and granules from pouches but wider for granules retrieved from the soil.

### 3.2. FTIR data

FTIR analyses revealed that in the milky fluid the $\nu_4$ peak at 714 cm$^{-1}$ was absent (Fig. 2). In contrast, the granules recovered from the pouch and from the soil both had a distinct peak at

714 cm$^{-1}$. The ratio of the peak areas for the $\nu_3$ and $\nu_4$ vibrations was significantly greater (*t*-test, p $\leq$ 0.05) for the pouch granules (31.9 $\pm$ 2.2, mean $\pm$ standard deviation, n = 3) than for the soil granules (21.3 $\pm$ 0.6).

## 4. Discussion

### 4.1. $\delta^{13}C$ values and polymorphs of $CaCO_3$

Ranges of $\delta^{13}$C values are narrow for the milky fluid and granules from pouches, while $\delta^{13}$C values for granules retrieved from the soil show a wide range and are not normally distributed (Fig. 1). Therefore, for each experimental replicate, the median $\delta^{13}$C value for each set of 10 granules recovered from the soil per earthworm was used for comparison with the $\delta^{13}$C values of the milky fluid and pouch granules recovered from the same earthworm that produced the soil granules. Regression analyses for the entire dataset (combining the different temperature treatments) revealed strong relationships between milky fluid $\delta^{13}$C values and $\delta^{13}$C values of the granules retrieved from the same earthworm's pouches (Fig. 3a), as well as between $\delta^{13}$C values of granules retrieved from the pouches and those secreted into the soil by the same earthworm (Fig. 3b). Relationships were less strong when individual temperature treatments were considered. Going from milky fluid, to granules in the pouches, to granules in the soil, $\delta^{13}$C values show a gradual shift to lower values (Fig. 1, Fig. 3a-b).

Previous studies show that the $CaCO_3$ in milky fluid mostly comprises ACC (Briones et al., 2008), while the granules mostly comprise calcite but with trace amounts of vaterite (Gago-Duport et al., 2008; Lee et al., 2008; Hodson et al., 2015). Our data are consistent with this. Our FTIR analyses (Fig. 2) indicate that the milky fluid was dominated by ACC (the $\nu_4$ peak at 714 cm$^{-1}$ was absent and the spectrum resembled that of the reference synthetic ACC). In contrast, the granules recovered from the pouch and from the soil both had a distinct peak at 714 cm$^{-1}$, typical of crystalline forms of $CaCO_3$ and a spectrum almost identical to reference calcite. However, we note that the FTIR spectrum of vaterite is almost identical to that of calcite (e.g. Hodson et al., 2015) and, in contrast to our previous studies, here we did not carry out the X-ray diffraction analysis necessary to confirm that the granules are calcite and not vaterite.

In previous studies relict ACC has been detected in granules (Gago-Duport et al., 2008; Lee et al., 2008; Fraser et al., 2011; Brinza et al., 2013, 2014a, b; Hodson et al., 2015). The ratio of the peak areas for the $\nu_3$ and $\nu_4$ vibrations was greater for the pouch granules than for the soil granules. The ratio of $\nu_3$ and $\nu_4$ decreases as the amount of ACC decreases (Hodson et al., 2015) suggesting that the granules from the pouches contain a larger amount of untransformed ACC than the granules recovered from the soil.

It is known that considerable $\delta^{13}$C fractionation factors exist between the different species of DIC and the various polymorphs of $CaCO_3$ (Romanek et al., 1992; Zhang et al., 1995; Szaran, 1997). The observed differences in $\delta^{13}$C values between milky fluid and the two types of granules could be due to analogous isotopic fractionation. Similarly Guiffre et al. (2015) observed a change in both the Ca and Mg isotopic composition of $CaCO_3$ as it transformed from ACC to calcite and attributed this to a dissolution-reprecipitation mechanism for the transformation. Thus, the observed C
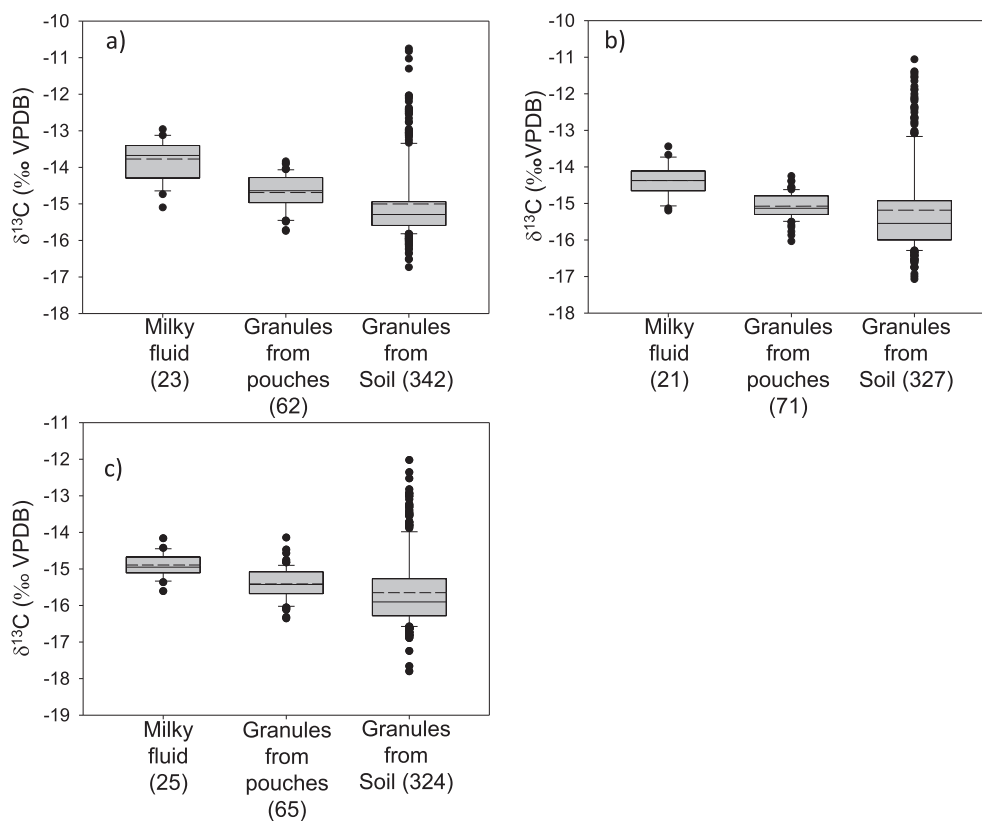


**Fig. 1.** Box plots showing the decrease in $\delta^{13}$C values for *L. terrestris*-produced milky fluid extracted from the granule-producing pouches, granules extracted from the pouches and $CaCO_3$ granules extracted from the soil for the a) 10 °C, b) 16 °C and c) 20 °C treatments. Values in brackets indicate sample numbers. Within each grey box the solid line represents the median value and the dashed line the mean. The top and bottom of the box define the 25th and 75th percentiles and the error bars the 5th and 95th percentiles. Data points which plot outside the 5th and 95th percentiles are plotted individually.
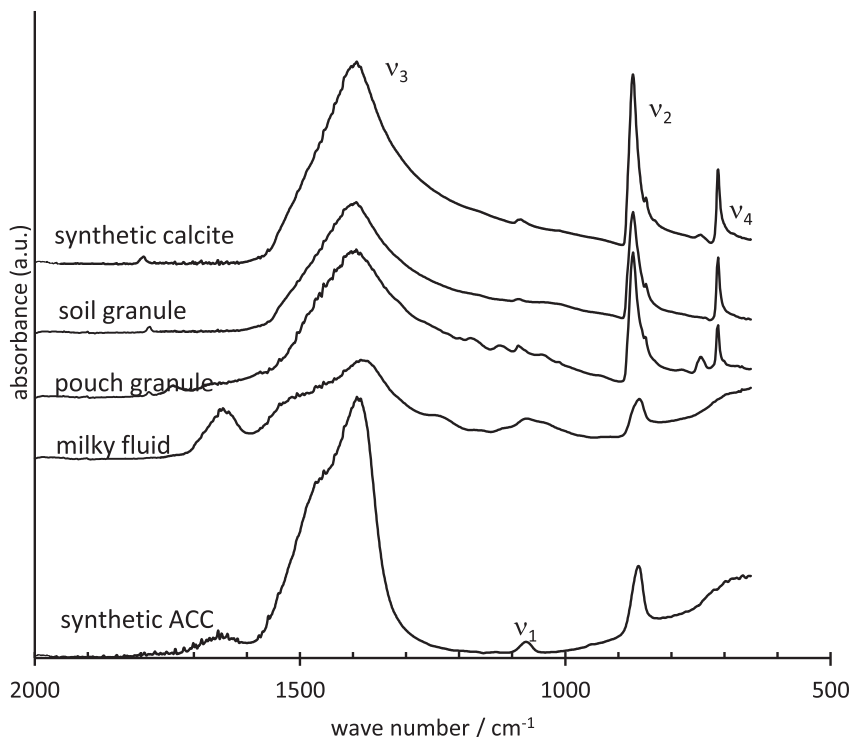
**Fig. 2.** Typical FTIR spectra for milky fluid, a granule recovered from one of the pouches (pouch granule), and a granule recovered from the soil (soil granule); all spectra relate to samples from the same earthworm. Reference spectra for synthetic calcite and ACC are also shown. Spectra are vertically offset on the absorbance axis for clarity. The major calcium carbonate peaks ($\upsilon_1$ to $\upsilon_4$) are labelled. Calcite shows peaks at ~714 $cm^{-1}$ ($\upsilon_4$), ~866 $cm^{-1}$ ($\upsilon_2$), ~1090 $cm^{-1}$ ($\upsilon_1$) and 1420–1470 $cm^{-1}$ ($\upsilon_3$); amorphous calcium carbonate lacks the ~714 $cm^{-1}$ peak.

fractionation reported here supports suggestions that the transformation of ACC into calcite occurs through dissolution and re-precipitation (e.g. Pontoni et al., 2003; Han and Aizenberg, 2008; Bots et al., 2012; Guiffre et al., 2015) rather than solid state dehydration and structural rearrangement (e.g. Beniash et al., 1999; Politi et al., 2008; Weiner and Addadi, 2011; Gal et al., 2013). As we only analysed samples by FTIR we are unable to comment on whether vaterite might form as an intermediate in this transformation.

Using the $\delta^{13}C$ values for the milky fluid, the pouch granules and median values for the soil-recovered granules for individual earthworms we estimated the isotopic enrichment factor ($\varepsilon$) between calcite (soil granules and pouch granules) and ACC, defined by:

$$\varepsilon_{calcite-ACC} = 1000 \cdot \left[ \left( \delta^{13}C_{calcite} + 1000 \right) \middle/ \left( \delta^{13}C_{ACC} + 1000 \right) - 1 \right]$$

(1)

The pouch granule - milky fluid ($-0.74 \pm 0.37$‰), soil granule - milky fluid ($-1.20 \pm 0.52$‰) and soil granule - pouch granule ($-0.46 \pm 0.45$‰) enrichment factors were significantly different from each other (Kruskal-Wallis One Way Analysis of Variance on Ranks followed by a post hoc Tukey test, $p \leq 0.01$) despite significant overlap between the first two. The high level of overlap between these two enrichment factors is undoubtedly due to the fact that the granules in the pouches and the soil are predominantly calcite. This also helps explain the soil granule − pouch granule enrichment factor that is almost equal to zero within error; the majority of ACC will have converted to calcite in the pouch granules and therefore little additional transformation occurs following

expulsion of the granules from the calciferous gland into the earthworm intestine and from there into the soil. Both the pouch granule - milky fluid and soil granule - milky fluid enrichment factors indicate an increase in the incorporation of $^{12}C$ relative to $^{13}C$ as the ACC crystallizes to calcite. This incorporation of the lighter isotope in the final crystallization product is in agreement with existing kinetic theories on the control of isotope fractionation (Watson, 2004; DePaolo, 2011; Nielsen et al., 2012) and has been observed in a variety of biominerals (e.g. Auclair et al., 2003; Rollion-Bard et al., 2016; Spooner et al., 2016) where the $\delta^{13}C$ of crystalline calcium carbonate is compared to that of dissolved precursor ions.

Although significant differences exist between the enrichment factors calculated for different temperatures for the pouch granule - milky fluid (ANOVA, $p \leq 0.01$) and soil granule - milky fluid (Kruskal-Wallis One Way Analysis of Variance on Ranks, $p \leq 0.01$) linear regression indicates only a small dependence of this variation on temperature ($R^2 < 0.2$), consistent with previous abiotic calcite-bicarbonate enrichment factors (Romanek et al., 1992), but not with theories considering kinetic controls on isotopic fractionation. The lack of an apparent temperature dependence may be due to either or both the metabolism of the earthworms maintaining a more constant body temperature in the calciferous gland than in the surrounding soil (though note that oxygen fractionation is temperature sensitive to the temperature of the surrounding soil, see Versteegh et al., 2013) or the scatter in the $\delta^{13}C$ values.

Guiffre et al. (2015) found that the Ca and Mg isotopic composition of calcite formed from ACC was sensitive to the amounts of ACC present. In a similar fashion, the relatively high standard deviation values for the enrichment factors may be due to a lack of end member ACC and calcite used in our calculation. Although the FTIR spectra for the milky fluid indicate that the only form of $CaCO_3$
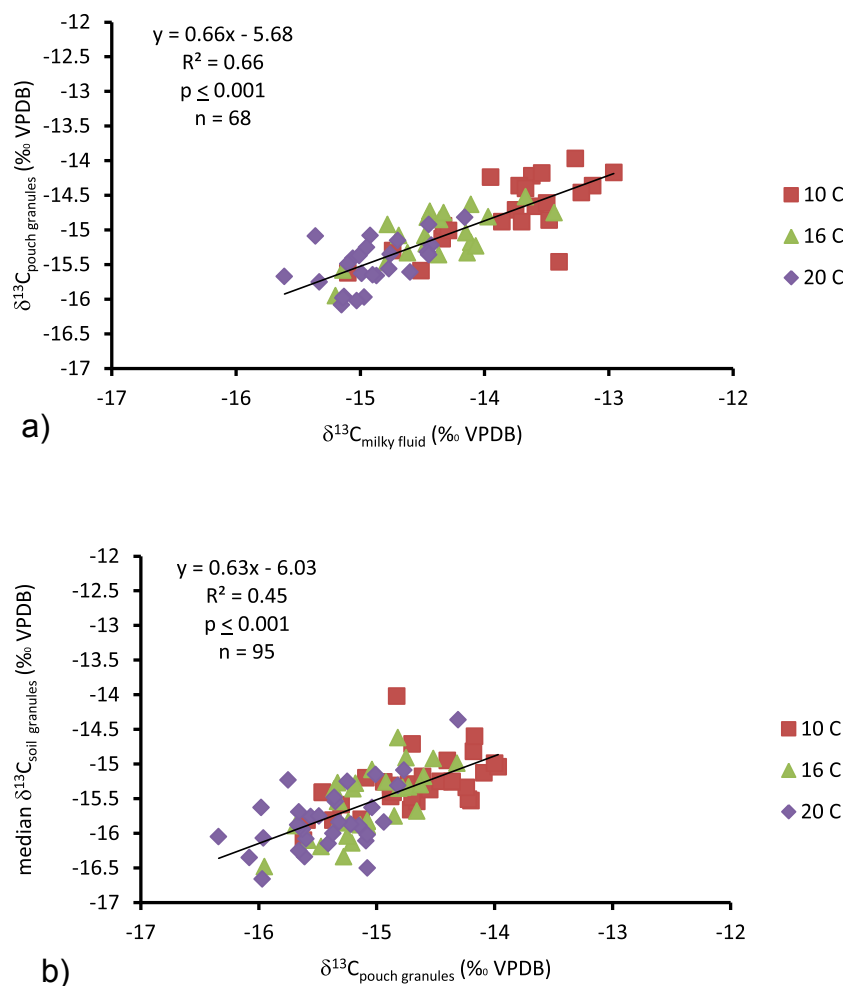
**Fig. 3.** (a) $\delta^{13}C$ values of individual granules retrieved from the pouches versus $\delta^{13}C$ values of milky fluid recovered from the same pouch, note the depletion in $^{13}C$ in the former and the shift to lower values with increasing temperature; (b) Median $\delta^{13}C$ composition of granules recovered from the soil versus $\delta^{13}C$ values of the individual granules retrieved from the pouches of the earthworm cultivated in that soil; note increased scatter and lower $R^2$. Data from all experiments is plotted.

present is ACC (Fig. 2), studies have found trace amounts of calcite in the milky fluid (Gago-Duport et al., 2008) and it is possible that that is the case here with the calcite below detection levels. Trace amounts of calcite may have been produced if some ACC transformed whilst the milky fluid was drying out. Our results suggest that this would result in more negative $\delta^{13}C$ values. Similarly the granules, although predominantly calcite, may contain varying, but small, amounts of ACC (e.g. Lee et al., 2008; Hodson et al., 2015). Small amounts of ACC appear to be unusually stable in the granules and may be preserved indefinitely in the granules. Further, granules recovered from the soil were secreted over a 28 day period and therefore potentially show different degrees of transformation from ACC to calcite. Varying levels of transformation from ACC to calcite from the milky fluid to the granules recovered from the soil are also consistent with the wider range of $\delta^{13}C$ values and enrichment factors observed for these granules compared to those present in the pouches which will have a more similar age and, therefore potentially have experienced the same amount of ACC transformation.

Methods have recently been developed to synthesise ACC that can remain stable for several days (Rodriguez-Blanco et al., 2008). This opens up the possibility of direct and accurate determination of $\varepsilon_{calcite-ACC}$ for abiotic systems in the near future. More detailed carbon isotope and mineralogical studies of the calciferous gland

and calcite granules together with other biominerals, e.g. echinoderm spines, in which both ACC and calcite are present are required to better understand the role of vital processes in this fractionation. As many biominerals are precipitated from an ACC precursor phase, and can contain stable ACC in their mature state, this will be an important step in understanding the mechanisms of biomineralisation and implications for the environmental interpretation of biomineral proxies.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.apgeochem.2017.01.017.

## References

Adkins, J.F., Boyle, E.A., Curry, W.B., Lutringer, A., 2003. Stable isotopes in deep-sea corals and a new mechanism for "vital effects". Geochim. Cosmochim. Acta 67, 1129–1143.

Aizenberg, J., Addadi, L., Weiner, S., Lambert, G., 1996. Stabilization of amorphous calcium carbonate by specialized macromolecules in biological and synthetic precipitates. Adv. Mater. 8, 222–226.

Aizenberg, J., Weiner, S., Addadi, L., 2003. Coexistence of amorphous and crystalline calcium carbonate in skeletal tissues. Connect. Tissue Res. 44, 20–25.

Aoki, K., 1934. Kalzium im blut des regenwurms. Proc. Imp. Acad. Jpn. 10, 121–124.

Auclair, A.-C., Joachmisky, M.M., Lécuyer, C., 2003. Deciphering kinetic, metabolic and environmental controls on stable isotope fractionations between seawater and the shell of *Terebratalia transversa*. Chem. Geol. 202, 59–78.

Avery, B.W., 1980. Soil Classification for England and Wales [higher Categories]. Rothamsted Experimental Station, Harpenden.

Beniash, E., Addadi, L., Weiner, S., 1999. Cellular control over spicule formation in sea urchin embryos: a structural approach. J. Struct. Biol. 125, 50–62.

Bernis, B.E., Spero, H.J., Lea, D.W., Bijma, J., 2000. Temperature influence on the carbon isotopic composition of *Globigerina bulloides* and *Orbulina universa* (planktonic foraminifera). Mar. Micropalaeonol. 38, 213–228.

Bots, P., Benning, L.G., Rodriguez-Blanco, J.-D., Roncal-Herrero, T., Shaw, S., 2012. Mechanistic insights into the crystallization of amorphous calcium carbonate (ACC). Cryst. growth Des. 12, 3806–3814.

Brinza, L., Quinn, P.D., Schofield, P.F., Mosselmans, J.F.W., Hodson, M.E., 2013. Incorporation of strontium in earthworm-secreted calcium carbonate granules produced in strontium-amended and strontium-bearing soil. Geochim. Cosmochim. Acta 113, 21–37.

Brinza, L., Schofield, P.F., Hodson, M.E., Weller, S., Ignatyev, K., Geraki, K., Quinn, P.D., Mosselmans, J.F.W., 2014a. Combining microxanes and microxrd mapping to analyse the heterogeneity in calcium carbonate granules excreted by the earthworm Lumbricus terrestris. J. Synchrotron Radiat. 21, 235–241.

Brinza, L., Schofield, P.F., Mosselmans, J.F.W., Donner, E., Lombi, E., Paterson, D., Hodson, M.E., 2014b. Can earthworm-secreted calcium carbonate immobilise Zn in contaminated soils? Soil Biol. Biochem. 74, 1–10.

Briones, M.J.I., López, E., Méndez, J., Rodríguez, J.B., Gago-Duport, L., 2008. Biological control over the formation and storage of amorphous calcium carbonate by earthworms. Mineral. Mag. 72, 227–231.

BS ISO, 1998. Soil Quality - Determination of the Water-retention Characteristic. BS ISO, Geneva, p. 24.

Canti, M.G., Piearce, T.G., 2003. Morphology and dynamics of calcium carbonate granules produced by different earthworm species. Pedobiologia 47, 511–521.

Chester, R., Elderfield, H., 1967. The application od infra-red absorption spectroscopy to carbonate mineralogy. Sedimentology 9, 5–21.

Darwin, C., 1881. The Formation of Vegetable Mould, through the Action of Worms, with Observations on Their Habits. The Echo Library, Teddington, p. 103.

DePaolo, D., 2011. Surface kinetic model for isotopic and trace element fractionation during precipitation of calcite from aqueous solutions. Geochim. Cosmochim. Acta 75, 1039–1056.

Fouke, B.W., Farmer, J.D., Des Marais, D.J., Pratt, L., Sturchio, N.C., Burns, P.C., Discipulo, M.K., 2000. Depositional facies and aqueous-solid geochemistry of travertine-depositing hot springs (Angle Terrace, Mammoth hot springs, Yellowstone National Park, USA). J. Sediment. Res. 70, 565–585.

Fraser, A., Lambkin, D.C., Lee, M.R., Schofield, P.F., Mosselmans, J.F.W., Hodson, M.E., 2011. Incorporation of lead into calcium carbonate granules secreted by earthworms living in lead contaminated soils. Geochim. Cosmochim. Acta 75, 2544–2556.

Gago-Duport, L., Briones, M.J.I., Rodríguez, J.B., Covelo, B., 2008. Amorphous calcium carbonate biomineralization in the earthworm's calciferous gland: pathways to the formation of crystalline phases. J. Struct. Biol. 162, 422–435.

Gal, A., Habraken, W., Gur, D., Fratzl, P., Weiner, S., Addadi, L., 2013. Calcite crystal growth by a solid-state transformation of stabilized amorphous calcium carbonate nanospheres in a hydrogel. Angew. Chem. Int. Ed. 52, 4867–4870.

Guiffre, A.J., Gagnon, A.C., de Yoreo, J.J., Dove, P.M., 2015. Isotopic tracer evidence for the amorphous calcium carbonate to calcite transformation by dissolution-reprecipitation. Geochim. Cosmochim. Acta 165, 407–417.

Han, T.Y.-J., Aizenberg, J., 2008. Calcium carbonate storage in amorphous form and its template-induced crystallization. Chem. Mater. 20, 1064–1068.

Hodson, M.E., Benning, L., Demarchi, B., Penkman, K., Rodriguez-Blanco, J.D., Schofield, P.F., Versteegh, E.A.A., 2015. Amorphous calcium carbonate stability in earthworm-secreted granules: an amino acid and synchrotron FTIR study. Geochem. Trans. 16, 4.

Jacob, D.E., Soldati, A.L., Wirth, R., Huth, J., Wehrmeister, U., Hofmeister, W., 2008. Nanostructure, composition and mechanisms of bivalve shell growth. Geochim. Cosmochim. Acta 72, 5401–5415.

Kaestner, A., 1967. Invertebrate Zoology. Interscience, New York.

Kühle, J.C., 1980. Vergleichende untersuchungen zur funktion der kalkdrüse verschiedener regenwurmarten bei unterschiedlicher $co_2$-atmosphäre. Verh. Ges. für Ökol. VIII, 411–415.

Lambkin, D.C., Gwilliam, K.H., Layton, C., Canti, M.G., Piearce, T.G., Hodson, M.E., 2011. Soil pH governs production rate of calcium carbonate secreted by the earthworm Lumbricus terrestris. Appl. Geochem. 26, S64–S66.

Lécuyer, C., Hutzler, A., Amiot, R., Daux, V., Grosheny, D., Otero, O., Martineau, F., Fourel, F., Balter, V., Reynard, B., 2012. Carbon and oxygen isotope fractionations between aragonite and calcite of shells from modern molluscs. Chem. Geol. 332–333, 92–101.

Lee, M.R., Hodson, M.E., Langworthy, G.N., 2008. Earthworms produce granules of intricately zoned calcite. Geology 36, 943–946.

McConnaughey, T., 1989. 13C and 18O isotopic disequilibrium in biological carbonates II: *In vitro* simulation of kinetic isotope effects. Geochim. Cosmochim. Acta 53, 163–171.

Nielsen, L.C., DePaolo, D.J., De Yoreo, J.J., 2012. Self-consistent ion-by-ion growth model for kinetic isotopic fractionation during calcite precipitation. Geochim. Cosmochim. Acta 86, 166–181.

Paul, D., Skrzypek, G., 2007. Assessment of carbonate-phosphoric acid analytical technique performed using gasbench ii in continuous flow isotope ratio mass spectrometry. Int. J. Mass Spectrom. 262, 180–186.

Politi, Y., Metzler, R.A., Abrecht, M., Gilbert, B., Wilt, F.H., Sagi, I., Addadi, L., Weiner, S., Gilbert, P.U.P.A., 2008. Transformation mechanism of amorphous calcium carbonate into calcite in sea urchin larval spicule. Proc. Natl. Acad. Sci. 105, 17362–17366.

Pontoni, D., Bolze, J., Dingenouts, N., Narayanan, T., Ballauff, M., 2003. Crystallization of calcium carbonate observed in-situ by combined small- and wide-angle X-ray scattering. J. Phys. Chem. B 107, 5123–5125.

Radha, A.V., Forbes, T.Z., Killian, C.E., Gilbert, P.U.P.A., Navrotsky, A., 2010. Transformation and crystallization energetics of synthetic and biogenic amorphous calcium carbonate. Proc. Natl. Acad. Sci. 107, 16438–16443.

Rodriguez-Blanco, J.D., Shaw, S., Benning, L.G., 2011. The kinetics and mechanisms of amorphous calcium carbonate (acc) crystallization to calcite, via vaterite. Nanoscale 3, 265–271.

Rodriguez-Blanco, J.D., Shaw, S., Benning, L.G., 2008. How to make "stable" ACC: protocol and preliminary structural characterization. Mineral. Mag. 72, 283–286.

Rollion-Bard, C., Saulnier, S., Vigier, N., Schumacher, A., Chaussidon, M., Lécuyer, C., 2016. Variability in magnesium, carbon and oxygen isotope compositions of brachiopod shells: implications for paleoceanographic studies. Chem. Geol. 423, 49–60.

Romanek, C.S., Grossman, E.L., Morse, J.W., 1992. Carbon isotopic fractionation in synthetic aragonite and calcite: effects of temperature and precipitation rate. Geochim. Cosmochim. Acta 56, 419–430.

Spooner, P.T., Guo, W.F., Robinson, L.F., Thiagarajan, N., Hendry, K.R., Rosenheim, B.E., Meng, M.J., 2016. Clumped isotope composition of cold-water corals: a role for vital effects? Geochim. Cosmochim. Acta 179, 123–141.

Stephens, C.J., Kim, Y.-Y., Evans, S.D., Meldrum, F.C., Christenson, H.K., 2011. Early stages of crystallization of calcium carbonate revealed in picoliter droplets. J. Am. Chem. Soc. 133, 5210–5213.

Szaran, J., 1997. Achievement of carbon isotope equilibrium in the system $HCO_3^-$ (solution)-$CO_2$(gas). Chem. Geol. 142, 79–86.

Versteegh, E.A.A., Black, S., Hodson, M.E., 2014. Environmental controls on the production of calcium carbonate by earthworms. Soil Biol. Biochem. 70, 159–161.

Versteegh, E.A.A., Black, S., Canti, M.G., Hodson, M.E., 2013. Earthworm secreted calcium carbonate – a new palaeothermometer? Geochim. Cosmochim. Acta 123, 351–357.

Voigt, O., 1933. Die funktion der regenwurm-kalkdrüsen. Zool. Jahrb. Abt. für Allg. Zool. Physiol. Tiere 52, 677–708.

Watson, B.E., 2004. A conceptual model for near-surface kinetic controls on the trace element and stable isotope composition of abiogenic calcite crystals. Geochim. Cosmochim. Acta 68, 1473–1488.

Wehrmeister, U., Jacob, D.E., Soldati, A.L., Loges, N., Häger, T., Hofmeister, W., 2011. Amorphous, nanocrystalline and crystalline calcium carbonates in biological materials. J. Raman Spectrosc. 42, 926–935.

Weiner, S., Addadi, L., 2011. Crystallization pathways in biomineralization. Annu. Rev. Mater. Res. 41, 21–40.

Zhang, J., Quay, P.D., Wilbur, D.O., 1995. Carbon isotope fractionation during gas-water exchange and dissolution of $co_2$. Geochim. Cosmochim. Acta 59, 107–114.