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

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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 $\varepsilon_{calcite\text{-}ACC} = -1.20 \pm 0.52‰$. 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.

1. Introduction

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

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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 ± 0.3 and 3.8 ± 0.1% for Hamble and 7.1 ± 0.1 and 7.4 ± 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 (±0.2) ‰ 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} (ν_4), ~866 cm^{-1} (ν_2), ~1084 cm^{-1} (ν_1) and 1420-1470 cm^{-1} (ν_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 ν_4 and ν_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 pair-wise 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 ν_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