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

E.A.A. Versteegh, S. Black, M.E. Hodson

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1 **Carbon isotope fractionation between amorphous calcium**

2 **carbonate and calcite in earthworm-produced calcium carbonate**

3 E. A. A. VERSTEEGH[1]*, S. BLACK[2], M. E. HODSON[1,3],**

4

5 [1] *University of Reading, Soil Research Centre, Department of Geography*

6 *and Environmental Science, School of Archaeology, Geography and*

7 *Environmental Science, Whiteknights P.O. Box 233, Reading RG6 6DW, UK*

8 [2] *University of Reading, Department of Archaeology, School of*

9 *Archaeology, Geography and Environmental Science, Whiteknights, P.O.*

10 *Box 227, Reading RG6 6AB, UK*

11 [3] *University of York, Environment Department, Heslington, York YO10*

12 *5DD, UK*

13

14 * E-mail: e.a.a.versteegh@gmail.com

15 ** E-mail: mark.hodson@york.ac.uk

16

17 **Abstract**

18 In this study we investigate carbon isotope fractionation during the

19 crystallization of biogenic calcium carbonate. Several species of

20 earthworm including *Lumbricus terrestris* secrete $CaCO_3$. Initially a

21 milky fluid comprising micro-spherules of amorphous $CaCO_3$ (ACC) is

22 secreted into pouches of the earthworm calciferous gland. The micro-

23   spherules coalesce and crystalize to form millimetre scale granules,

24   largely comprising calcite. These are secreted into the earthworm

25   intestine and from there into the soil. *L. terrestris* were cultured for 28

26   days in two different soils, moistened with three different mineral

27   waters at 10, 16 and 20 °C. The milky fluid in the calciferous glands,

28   granules in the pouches of the calciferous glands and granules excreted

29   into the soil were collected and analysed by FTIR spectroscopy to

30   determine the form of $CaCO_3$ present and by IRMS to determine $\delta^{13}C$

31   values. The milky fluid was ACC. Granules removed from the pouches

32   and soil were largely calcite; the granules removed from the pouches

33   contained more residual ACC than those recovered from the soil. The

34    $\delta^{13}C$ values of milky fluid and pouch granules became significantly more

35   negative with increasing temperature ($p \leq 0.001$). For samples from

36   each temperature treatment, $\delta^{13}C$ values became significantly ($p \leq$

37   0.001) more negative from the milky fluid to the pouch granules to the

38   soil granules (-13.77, -14.69 and -15.00 respectively at 10 °C; -14.37, -

39   15.07 and -15.18 respectively at 16 °C and -14.89, -15.41 and -15.65

40   respectively at 20 °C). Fractionation of C isotopes occurred as the ACC

41   recrystallized to form calcite with the fractionation factor $\varepsilon_{calcite\text{-}ACC}$ = -

42   1.20 ± 0.52 ‰. This is consistent with the crystallization involving

43   dissolution and reprecipitation rather than a solid state rearrangement.

44   Although C isotopic fractionation has previously been described

2

45   between different species of dissolved inorganic carbon and various

46   CaCO$_3$ polymorphs, this is the first documented evidence for C isotope

47   fractionation between ACC and the calcite it recrystallizes to. This

48   phenomenon may prove important for the interpretation of CaCO$_3$-

49   based C isotope environmental proxies.

50

51   **Keywords**: Earthworms; calcium carbonate; calcite; carbon isotopes;

52   fractionation; crystallization

53

54   **Introduction**

55   Many earthworm species produce calcium carbonate (CaCO$_3$) granules

56   in specialised calciferous glands. In the earthworm *Lumbricus terrestris*

57   these occur in segments 11-12 as two pairs of swellings off the

58   oesophagus, and one pair of pouches anterior to the glands in segment

59   10 (Darwin, 1881; Canti and Piearce, 2003).

60   CaCO$_3$ production starts by secretion of an amorphous calcium

61   carbonate (ACC) suspension that we refer to as milky fluid. In the

62   pouches, small spherulites (1-5 μm) in the milky fluid accrete into

63   larger granules (≤ 2.5 mm). These are released into the oesophagus and

64   excreted into the soil (Briones *et al.*, 2008; Gago-Duport *et al.*, 2008).

65   The granules retrieved from the pouches and the soil are

66   predominantly calcite, but can contain small amounts of ACC, vaterite

67   and aragonite (Gago-Duport *et al.*, 2008; Lee *et al.*, 2008; Fraser *et al.*,

68   2011; Brinza *et al.*, 2013; Brinza *et al.*, 2014a; Brinza *et al.*, 2014b;

69   Hodson et al., 2015). The function of $CaCO_3$ production by the

70   earthworms remains unclear but is likely related to regulation of pH

71   and CO_2 concentrations in body fluids (Voigt, 1933; Aoki, 1934;

72   Kaestner, 1967; Kühle, 1980; Versteegh *et al.*, 2014).

73   It is known that considerable $\delta^{13}C$ fractionation factors exist between

74   the different species of DIC and the various polymorphs of $CaCO_3$

75   (Fouke et al., 2000; Romanek *et al.*, 1992; Szaran, 1997; Zhang *et al.*,

76   1995). In addition to thermodynamics, kinetics of precipitation plays an

77   important role in fractionation (Watson, 2004; DePaolo, 2011; Nielsen

78   et al., 2012). Variable fractionation of carbon isotopes has been

79   observed in different calcium carbonate biominerals suggesting that

80   vital effects may also be relevant (e.g. Adkins et al., 2003; Auclair et al.,

81   2003; Bernis et al. 2000; Lécuyer et al. 2012; McConnaughey, 1989;

82   Rollion-Bard et al, 2016; Spooner et al, 2016). Despite many calcium

83   carbonate minerals having an amorphous pre-cursor (Radha *et al.*,

84   2010; Rodriguez-Blanco *et al.*, 2011; Stephens *et al.*, 2011) and stable

85   ACC being increasingly observed in biominerals (Aizenberg *et al.*, 2003;

86   Jacob *et al.*, 2008; Wehrmeister *et al.*, 2011), carbon isotope

87   fractionation between ACC and calcite has not been previously reported

88   in the literature

4

89   Here we present results of stable carbon isotope analyses on milky

90   fluid collected from the calciferous pouches of earthworms, fresh

91   granules also collected from the pouches, and older granules collected

92   from the soil in which the earthworms were cultivated and address the

93   question: how does granule mineralogy influence $\delta^{13}C$ values?

94   Furthermore we make a first attempt at estimating the carbon isotopic

95   fractionation factor between calcite and ACC, produced by earthworms.

96

97   **Materials & Methods**

98   *Experimental setup*

99   Two soils were collected from agricultural fields in Berkshire, UK:

100   Hamble (SU 61968 70235) and Red Hill (SU 56060 80033); both

101   Typical Argillic Brown Earths (Avery, 1980; full soil characterisation in

102   Table 1, Versteegh et al., 2014). The soil was air-dried and sieved to 250

103   μm prior to use (Lambkin *et al.*, 2011). This ensures that no large

104   granules are present in the soil at the beginning of the experiment and

105   facilitates granule recovery at the end. Post-sieving soil pH and organic

106   matter content were 7.5 ± 0.3 and 3.8 ± 0.1 % for Hamble and 7.1 ± 0.1

107   and 7.4 ± 0.1 % for Red Hill. For each replicate, 300 g of soil were mixed

108   with one of three types of mineral water (initial $\delta^{18}O$ values -10.0, -7.3

109   and -5.3 (± 0.2) ‰ VSMOW) to 65 % water holding capacity (BS ISO,

110   1998). The moistened soil was put in a zip-lock bag with 5 g air-dried

5

111   horse manure rehydrated with 10 ml demineralised water. One adult,

112   clitellate *L. terrestris* was added to each bag. Bags were closed and kept

113   at either 10, 16 or 20 °C. There were six replicates per treatment.

114   Earthworms were acclimatised for three weeks, and then transferred to

115   an identical treatment bag containing the same type and mass of soil

116   and manure at the same temperature. Experimental details are given in

117   Versteegh et al. (2013). After 28 days earthworms were removed from

118   the bags, killed by dipping them in near-boiling water, and the

119   calciferous glands were dissected out. Any $CaCO_3$ concretions present in

120   the pouches were also retrieved, rinsed in deionised water and air-

121   dried. Calciferous glands were put on a glass slide; MF was allowed to

122   leak from the glands, was left to air-dry overnight, and collected by

123   scraping it off the slide. The soil was wet-sieved to 500 μm to retrieve

124   granules which were air-dried.

125

126   *Stable-isotope analyses*

127   Milky fluid and individual granule $CaCO_3$ samples were analysed for

128     $\delta^{13}C$ values using a Thermo Delta V Advantage IRMS with a GasBench II.

129   The Gasbench II sample preparation device uses 100% ortho-

130   phosphoric acid to transform $CaCO_3$ into CO_2 and hence only analyses

131   the mineral fraction of the samples (Paul and Skrzypek, 2007). The raw

132     $\delta^{13}C$ values were converted to the VPDB scale after normalising against

6

133   NBS 18 and NBS 19 carbonate standards. The long-term standard

134   deviation of a routinely analysed in-house $CaCO_3$ standard was < 0.05

135   ‰. Statistical analysis of the ^{13}C data was carried out using SigmaPlot

136   12 for Windows 7.

137

138   *Fourier transform infrared spectroscopy (FTIR)*

139   Three samples each of milky fluid, granules from pouches and granules

140   from soil were analysed by FTIR in the range 650 – 4000 cm^{-1} using a

141   diamond internal reflection cell on a A2-Technology MicroLab Portable

142   mid-IR spectrometer of the Cohen Laboratories, University of Leeds.

143   Spectra were acquired by co-adding 512 scans with a 4 cm^{-1} resolution.

144   Crystalline carbonate phases have distinct bands at $\sim$ 714 cm^{-1} (ν_4), $\sim$

145   866 cm^{-1} (ν_2), $\sim$ 1084 cm^{-1} (ν_1) and 1420-1470 cm^{-1} (ν_3) whilst ACC

146   lacks the distinct vibrational band at $\sim$ 714 cm^{-1} (Chester and

147   Elderfield, 1967; Aizenberg *et al.*, 1996; Gago-Duport *et al.*, 2008;

148   Rodriguez-Blanco *et al.*, 2011). Areas for the ν_4 and ν_3 peaks covering

149   the wavenumber ranges between 651 – 725 cm^{-1} and 1602-1243 cm^{-1}

150   respectively were determined using the Nicolet EZ OMNIC 5.1 Software.

151   Reference spectra for synthetic calcite and ACC were provided by Dr.

152   Juan-Diego Rodriguez-Blanco, University of Copenhagen, Department of

153   Chemistry.

154

155 **Results**

156 *$\delta^{13}C$ values of $CaCO_3$*

157 For each individual earthworm, 10 granules were analysed from the soil

158 and one from each of the pouches (if available). For milky fluid, only one

159 analysis per earthworm could be undertaken, but sometimes this failed

160 because too little material was available. All analyses are reported in

161 the Supplementary material. Three-way Analysis of Variance (ANOVA)

162 with temperature, soil type and water type as factors indicated that

163 there were no significant differences in $\delta^{13}C$ values of granules

164 extracted from the soil between different treatments. In contrast, 3-way

165 ANOVA followed by pair-wise multiple comparison (Holm-Sidak

166 method) indicated that there were significant differences in $\delta^{13}C$ values

167 between different temperature treatments for the granules extracted

168 from the pouches and also for the milky fluid ($p \leq 0.01$); values became

169 increasingly negative from the 10 to 16 to 20 °C treatments. There were

170 no significant differences in $\delta^{13}C$ values for either the milky fluid or

171 granules from pouches between different soils or different mineral

172 water treatments. Consequently, the data for different soil-water

173 combinations but the same temperature were combined for analysis.

174 Kruskall-Wallis One-way Analysis of Variance (ANOVA) on ranks

175 followed by pair-wise comparison (Dunn's method) indicated that at

176 each temperature there were significant differences between the $\delta^{13}C$

177   values of the milky fluid, granules from pouches and granules from soil

178   with values becoming increasingly negative in that order (Fig. 1).

179   Ranges of $\delta^{13}C$ values were relatively narrow for milky fluid and

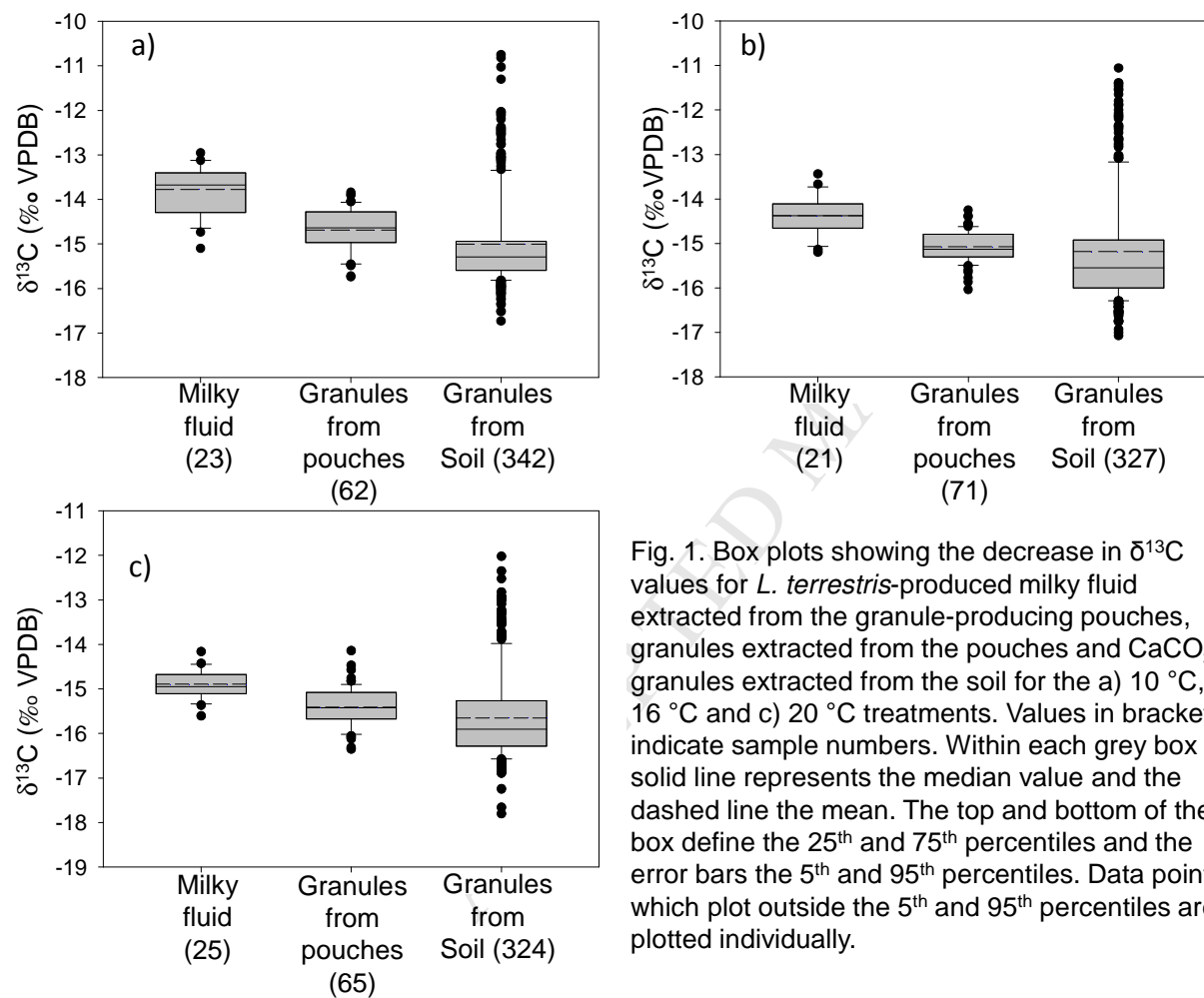180   granules from pouches but wider for granules retrieved from the soil.

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.

181

10

182

183 *FTIR data*

184 FTIR analyses revealed that in the milky fluid the ν_4 peak at 714 cm^{-1}

185 was absent (Fig. 2). In contrast, the granules recovered from the pouch

186 and from the soil both had a distinct peak at 714 cm^{-1}. The ratio of the

187 peak areas for the ν_3 and ν_4 vibrations was significantly greater (t-test,

188 $p \leq 0.05$) for the pouch granules (31.9 ± 2.2 ,mean $\pm$ standard deviation,

189 n = 3) than for the soil granules (21.3 ± 0.6).

190
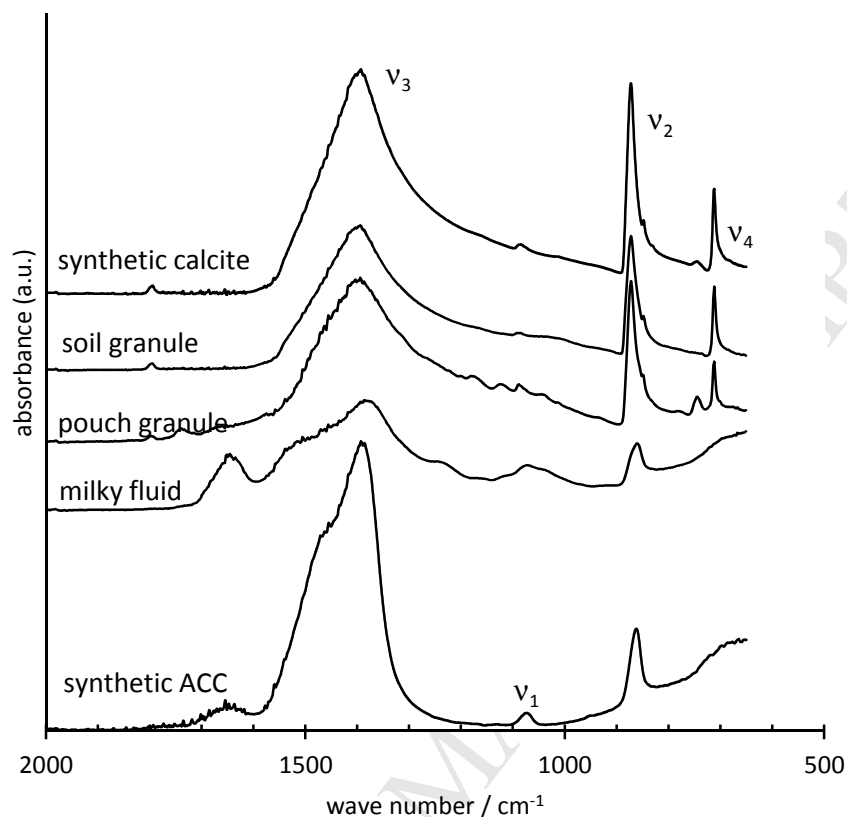
191

192　Fig. 2. Typical FTIR spectra for milky fluid, a granule recovered from
193　one of the pouches (pouch granule), and a granule recovered from the
194　soil (soil granule);all spectra relate to samples from the same
195　earthworm. Reference spectra for synthetic calcite and ACC are also
196　shown. Spectra are vertically offset on the absorbance axis for clarity.
197　The major calcium carbonate peaks (υ_1 to υ_4) are labelled. Calcite
198　shows peaks at $\sim$714 cm^{-1} (υ_4), $\sim$866 cm^{-1} (υ_2), $\sim$1090 cm^{-1} (υ_1) and
199　1420-1470 cm^{-1} (υ_3); amorphous calcium carbonate lacks the $\sim$714 cm^{-}
200　^{1} peak.
201

Discussion

203　*$\delta^{13}C$ values and polymorphs of $CaCO_3$*

204　Ranges of $\delta^{13}C$ values are narrow for the milky fluid and granules from

205　pouches, while $\delta^{13}C$ values for granules retrieved from the soil show a

12

206 wide range and are not normally distributed (Fig. 1). Therefore, for

207 each experimental replicate, the median $\delta^{13}C$ value for each set of 10

208 granules recovered from the soil per earthworm was used for

209 comparison with the $\delta^{13}C$ values of the milky fluid and pouch granules

210 recovered from the same earthworm that produced the soil granules.

211 Regression analyses for the entire dataset (combining the different

212 temperature treatments) revealed strong relationships between milky

213 fluid $\delta^{13}C$ values and $\delta^{13}C$ values of the granules retrieved from the

214 same earthworm's pouches (Fig. 3a), as well as between $\delta^{13}C$ values of

215 granules retrieved from the pouches and those secreted into the soil by

216 the same earthworm (Fig. 3b). Relationships were less strong when

217 individual temperature treatments were considered. Going from milky

218 fluid, to granules in the pouches, to granules in the soil, $\delta^{13}C$ values

219 show a gradual shift to lower values (Fig. 1, Fig. 3a-b).

220

a)

221

b)

222
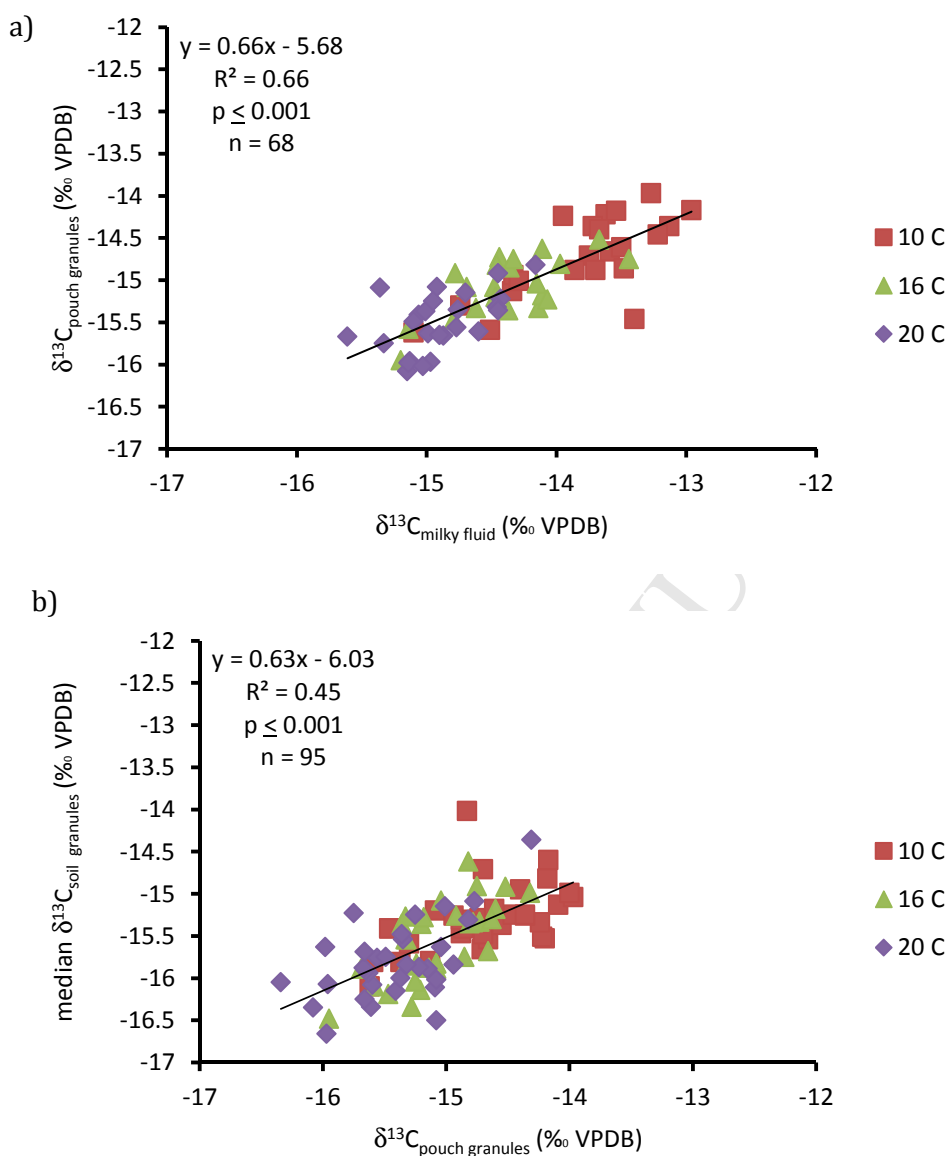
223 FIG. 3. (*a*) $\delta^{13}C$ values of individual granules retrieved from the pouches
224 versus $\delta^{13}C$ values of milky fluid recovered from the same pouch, note
225 the depletion in ^{13}C in the former and the shift to lower values with
226 increasing temperature; (*b*) Median $\delta^{13}C$ composition of granules
227 recovered from the soil versus $\delta^{13}C$ values of the individual granules
228 retrieved from the pouches of the earthworm cultivated in that soil;
229 note increased scatter and lower R^2. Data from all experiments is
230 plotted.
231

14

232   Previous studies show that the $CaCO_3$ in milky fluid mostly

233   comprises ACC (Briones *et al.*, 2008), while the granules mostly

234   comprise calcite but with trace amounts of vaterite (Gago-Duport *et al.*,

235   2008; Lee *et al.*, 2008; Hodson *et al.*, 2015). Our data are consistent with

236   this. Our FTIR analyses (Fig. 2) indicate that the milky fluid was

237   dominated by ACC (the v_4 peak at 714 cm^{-1} was absent and the

238   spectrum resembled that of the reference synthetic ACC). In contrast,

239   the granules recovered from the pouch and from the soil both had a

240   distinct peak at 714 cm^{-1}, typical of crystalline forms of $CaCO_3$ and a

241   spectrum almost identical to reference calcite, though we note that the

242   FTIR spectrum of vaterite is almost identical to that of calcite (e.g.

243   Hodson *et al.*, 2015) and, in contrast to our previous studies, here we

244   did not carry out the X-ray diffraction data necessary to confirm that the

245   granules are calcite and not vaterite.

246   In previous studies relict ACC has been detected in granules (Gago-

247   Duport *et al.*, 2008; Lee *et al.* 2008; Fraser *et al.*, 2011; Brinza *et al.*,

248   2013, 2014a, b; Hodson *et al.*, 2015). The ratio of the peak areas for the

249   v_3 and v_4 vibrations was greater for the pouch granules than for the soil

250   granules. The ratio of v_3 and v_4 decreases as the amount of ACC

251   decreases (Hodson *et al.*, 2015) suggesting that the granules from the

252   pouches contain a larger amount of untransformed ACC than the

253   granules recovered from the soil.

15

254 It is known that considerable $\delta^{13}C$ fractionation factors exist between

255 the different species of DIC and the various polymorphs of $CaCO_3$

256 (Romanek *et al.*, 1992; Zhang *et al.*, 1995; Szaran, 1997). The observed

257 differences in $\delta^{13}C$ values between milky fluid and the two types of

258 granules could be due to analogous isotopic fractionation. Similarly

259 Guiffre et al (2015) observed a change in both the Ca and Mg isotopic

260 composition of $CaCO_3$ as it transformed from ACC to calcite and

261 attributed this to a dissolution-reprecipitation mechanism for the

262 transformation. Thus, the observed C fractionation reported here

263 supports suggestions that the transformation of ACC into calcite occurs

264 through dissolution and re-precipitation (e.g. Pontoni *et al.*, 2003; Han

265 and Aizenberg, 2008; Bots *et al.*, 2012; Guiffre *et al.*, 2015) rather than

266 solid state dehydration and structural rearrangement (e.g. Beniash *et*

267 *al.*, 1999; Politi *et al.*, 2008; Weiner and Addabi, 2011; Gal *et al.*, 2013).

268 As we only analysed samples by FTIR we are unable to comment on

269 whether vaterite might form as an intermediate in this transformation.

270 Using the $\delta^{13}C$ values for the milky fluid, the pouch granules and

271 median values for the soil-recovered granules for individual

272 earthworms we estimated the isotopic enrichment factor (ε) between

273 calcite (soil granules and pouch granules) and ACC, defined by:

274

275 $\varepsilon_{calcite\text{-}ACC} = 1000 \cdot [(\delta^{13}C_{calcite} + 1000)/(\delta^{13}C_{ACC} + 1000) - 1]$ (1)

16

276

277   The pouch granule - milky fluid (-0.74 ± 0.37 ‰), soil granule - milky

278   fluid (-1.20 ± 0.52 ‰) and soil granule - pouch granule (-0.46 ± 0.45

279   ‰) enrichment factors were significantly different from each other

280   (Kruskal-Wallis One Way Analysis of Variance on Ranks followed by a

281   post hoc Tukey test, p ≤ 0.01) despite significant overlap between the

282   first two. The high level of overlap between these two enrichment

283   factors is undoubtedly due to the fact that the granules in the pouches

284   and the soil are predominantly calcite. This also helps explain the soil

285   granule – pouch granule enrichment factor that is almost equal to zero

286   within error; the majority of ACC will have converted to calcite in the

287   pouch granules and therefore little additional transformation occurs

288   following expulsion of the granules from the calciferous gland into the

289   earthworm intestine and from there into the soil. Both the pouch

290   granule - milky fluid and soil granule - milky fluid enrichment factors

291   indicate an increase in the incorporation of ^{12}C relative to ^{13}C as the ACC

292   crystallizes to calcite. This incorporation of the lighter isotope in the

293   final crystallization product is in agreement with existing kinetic

294   theories on the control of isotope fractionation (Watson, 2004; DePaolo,

295   2011; Nielsen et al., 2012) and has been observed in a variety of

296   biominerals (e.g. Auclair et al., 2003; Rollion-Bard et al, 2016; Spooner

17

297 et al, 2016) where the $\delta^{13}C$ of crystalline calcium carbonate is compared

298 to that of dissolved precursor ions.

299   Although significant differences exist between the enrichment

300 factors calculated for different temperatures for the pouch granule -

301 milky fluid (ANOVA, $p \leq 0.01$) and soil granule - milky fluid (Kruskal-

302 Wallis One Way Analysis of Variance on Ranks, $p \leq 0.01$) linear

303 regression indicates only a small dependence of this variation on

304 temperature ($R^2 < 0.2$), consistent with previous abiotic calcite-

305 bicarbonate enrichment factors (Romanek et al., 1992), but not with

306 theories considering kinetic controls on isotopic fractionation. The lack

307 of an apparent temperature dependence may be due to either or both

308 the metabolism of the earthworms maintaining a more constant body

309 temperature in the calciferous gland than in the surrounding soil

310 (though note that oxygen fractionation is temperature sensitive to the

311 temperature of the surrounding soil, see Versteegh et al., 2013) or the

312 scatter in the $\delta^{13}C$ values.

313   Guiffre *et al.* (2015) found that the Ca and Mg isotopic composition of

314 calcite formed from ACC was sensitive to the amounts of ACC present. In

315 a similar fashion, the relatively high standard deviation values for the

316 enrichment factors may be due to a lack of end member ACC and calcite

317 used in our calculation. Although the FTIR spectra for the milky fluid

318 indicate that the only form of $CaCO_3$ present is ACC (Fig. 2), studies have

18

319   found trace amounts of calcite in the milky fluid (Gago-Duport *et al.*,

320   2008) and it is possible that that is the case here with the calcite below

321   detection levels. Trace amounts of calcite may have been produced if

322   some ACC transformed whilst the milky fluid was drying out. Our

323   results suggest that this would result in more negative $\delta^{13}C$ values.

324   Similarly the granules, although predominantly calcite, may contain

325   varying, but small, amounts of ACC (e.g. Lee *et al.*, 2008; Hodson *et al.*,

326   2015)). Small amounts of ACC appear to be unusually stable in the

327   granules and may be preserved indefinitely in the granules. Further,

328   granules recovered from the soil were secreted over a 28 day period

329   and therefore potentially show different degrees of transformation

330   from ACC to calcite. Varying levels of transformation from ACC to calcite

331   from the milky fluid to the granules recovered from the soil are also

332   consistent with the wider range of $\delta^{13}C$ values and enrichment factors

333   observed for these granules compared to those present in the pouches

334   which will have a more similar age and, therefore potentially have

335   experienced the same amount of ACC transformation.

336   Methods have recently been developed to synthesise ACC that can

337   remain stable for several days (Rodriguez-Blanco et al., 2008). This

338   opens up the possibility of direct and accurate determination of $\varepsilon_{\text{calcite-}}$

339    $_{\text{ACC}}$ for abiotic systems in the near future. More detailed carbon isotope

340   and mineralogical studies of the calciferous gland and calcite granules

19

341 together with other biominerals, e.g. echinoderm spines, in which both

342 ACC and calcite are present are required to better understand the role

343 of vital processes in this fractionation. As many biominerals are

344 precipitated from an ACC precursor phase, and can contain stable ACC

345 in their mature state, this will be an important step in understanding

346 the mechanisms of biomineralisation and implications for the

347 environmental interpretation of biomineral proxies.

348

362

References

363   Adkins, J.F., Boyle, E.A., Curry, W.B. and Lutringer, A. (2003) Stable

364   isotopes in deep-sea corals and a new mechanism for "vital effects".

365   *Geochim Cosmochim. Acta*, **67**, 1129-1143.

366   Aizenberg, J., Addadi, L., Weiner, S. and Lambert, G. (1996) Stabilization

367   of amorphous calcium carbonate by specialized macromolecules in

368   biological and synthetic precipitates. *Advanced Materials*, **8**, 222-226.

369   Aizenberg, J., Weiner, S. and Addadi, L. (2003) Coexistence of

370   amorphous and crystalline calcium carbonate in skeletal tissues.

371   *Connective Tissue Research*, **44**, 20-25.

372   Aoki, K. (1934) Kalzium im blut des regenwurms. *Proceedings of the*

373   *Imperial Academy of Japan*, **10**, 121-124.

374   Auclair, A.-C., Joachmisky, M.M., Lécuyer, C. (2003) Deciphering kinetic,

375   metabolic and environmental controls on stable isotope

376   fractionations between seawater and the shell of *Terebratalia*

377   *transversa*. *Chemical Geol*ogy, **202**, 59–78.

378   Avery, B.W. (1980) *Soil classification for England and Wales [higher*

379   *categories]*. Rothamsted Experimental Station, Harpenden.

380   Beniash, E., Addadi, L. and Weiner, S. (1999) Cellular control over

381   spicule formation in sea urchin embryos: a structural approach.

382   *Journal of Structural Biology,* **125**, 50-62.

21

384 Bernis, B.E., Spero, H.J., Lea, D.W. and Bijma, J. (2000) Temperature

385 influence on the carbon isotopic composition of *Globigerina bulloides*

386 and *Orbulina universa* (planktonic foraminifera) *Marine*

387 *Micropalaeonology*, **38**, 213-228.

388 Bots, P., Benning, L. G., Rodriguez-Blanco, J.-D., Roncal-Herrero, T. and

389 Shaw, S. (2012) Mechanistic insights into the crystallization of

390 amorphous calcium carbonate (ACC). *Crystal growth and Design*, **12**,

391 3806-3814.

392 Brinza, L., Quinn, P.D., Schofield, P.F., Mosselmans, J.F.W. and Hodson,

393 M.E. (2013) Incorporation of strontium in earthworm-secreted

394 calcium carbonate granules produced in strontium-amended and

395 strontium-bearing soil. *Geochimica et Cosmochimica Acta*, **113**, 21-

396 37.

397 Brinza, L., Schofield, P.F., Hodson, M.E., Weller, S., Ignatyev, K., Geraki, K.,

398 Quinn, P.D. and Mosselmans, J.F.W. (2014a) Combining microxanes

399 and microxrd mapping to analyse the heterogeneity in calcium

400 carbonate granules excreted by the earthworm *Lumbricus terrestris*.

401 *Journal of Synchrotron Radiation*, **21**, 235-241.

402 Brinza, L., Schofield, P.F., Mosselmans, J.F.W., Donner, E., Lombi, E.,

403 Paterson, D. and Hodson, M.E. (2014b) Can earthworm-secreted

404 calcium carbonate immobilise Zn in contaminated soils? *Soil Biology*

405 *and Biochemistry*, **74**, 1-10.

406   Briones, M.J.I., López, E., Méndez, J., Rodríguez, J.B. and Gago-Duport, L.

407   (2008) Biological control over the formation and storage of

408   amorphous calcium carbonate by earthworms. *Mineralogical*

409   *Magazine*, **72**, 227-231.

410   BS ISO. (1998) *Soil quality - determination of the water-retention*

411   *characteristic*. Pp. 24. BS ISO, Geneva.

412   Canti, M.G. and Piearce, T.G. (2003) Morphology and dynamics of

413   calcium carbonate granules produced by different earthworm

414   species. *Pedobiologia*, **47**, 511-521.

415   Chester, R. and Elderfield, H. (1967) The application od infra-red

416   absorption spectroscopy to carbonate mineralogy. *Sedimentology*, **9**,

417   5-21.

418   Darwin, C. (1881) *The formation of vegetable mould, through the action*

419   *of worms, with observations on their habits*. Pp. 103. The Echo

420   Library, Teddington.

421   DePaolo, D., 2011. Surface kinetic model for isotopic and trace element

422   fractionation during precipitation of calcite from aqueous solutions.

423   *Geochimica et Cosmochimica Acta*, **75**, 1039–1056.

424   Fouke, B.W., Farmer, J.D., Des Marais, D.J., Pratt, L., Sturchio, N.C., Burns,

425   P.C. and Discipulo, M.K. (2000) Depositional facies and aqueous-solid

426   geochemistry of travertine-depositing hot springs (Angle Terrace,

427   Mammoth Hot Springs, Yellowstone National Park, USA). *Journal of*

428   *Sedimentary Research,* 70, 565-585.

429   Fraser, A., Lambkin, D.C., Lee, M.R., Schofield, P.F., Mosselmans, J.F.W.

430   and Hodson, M.E. (2011) Incorporation of lead into calcium

431   carbonate granules secreted by earthworms living in lead

432   contaminated soils. *Geochimica et Cosmochimica Acta*, **75**, 2544-

433   2556.

434   Gago-Duport, L., Briones, M.J.I., Rodríguez, J.B. and Covelo, B. (2008)

435   Amorphous calcium carbonate biomineralization in the earthworm's

436   calciferous gland: Pathways to the formation of crystalline phases.

437   *Journal of Structural Biology*, **162**, 422-435.

438   Gal, A., Habraken, W., Gur, D., Fratzl, P., Weiner, S. and Addadi, L. (2013)

439   Calcite crystal growth by a solid-state transformation of stabilized

440   amorphous calcium carbonate nanospheres in a hydrogel.

441   *Angewandte Chemie International Edition*, **52**, 4867-4870.

442   Guiffre, A.J., Gagnon, A.C., de Yoreo, J.J. and Dove, P.M. (2015) Isotopic

443   tracer evidence for the amorphous calcium carbonate to calcite

444   transformation by dissolution-reprecipitation. *Geochimica et*

445   *Cosmochimica Acta*, **165**, 407-417.

446   Han, T. Y.-J. and Aizenberg, J. (2008) Calcium carbonate storage in

447   amorphous form and its template-induced crystallization. *Chemical*

448   *Materials*, **20**, 1064-1068.

449   Hodson, M.E., Benning, L., Demarchi, B., Penkman, K., Rodriguez-Blanco,

450   J.D., Schofield, P.F., Versteegh, E.A.A. (2015) Amorphous calcium

451   carbonate stability in earthworm-secreted granules: an amino acid

452   and synchrotron FTIR study. *Geochemical Transactions* **16**: 4.

453   Jacob, D.E., Soldati, A.L., Wirth, R., Huth, J., Wehrmeister, U. and

454   Hofmeister, W. (2008) Nanostructure, composition and mechanisms

455   of bivalve shell growth. *Geochimica et Cosmochimica Acta*, **72**, 5401-

456   5415.

457   Kaestner, A. (1967) *Invertebrate zoology*. Interscience, New York.

458   Kühle, J.C. (1980) Vergleichende untersuchungen zur funktion der

459   kalkdrüse verschiedener regenwurmarten bei unterschiedlicher CO_2-

460   atmosphäre. *Verhandlungen der Gesellschaft für Ökologie*, **VIII**, 411-

461   415.

462   Lambkin, D.C., Gwilliam, K.H., Layton, C., Canti, M.G., Piearce, T.G. and

463   Hodson, M.E. (2011) Soil pH governs production rate of calcium

464   carbonate secreted by the earthworm *Lumbricus terrestris*. *Applied

465   Geochemistry*, **26**, S64-S66.

466   Lécuyer, C., Hutzler, A., Amiot, R., Daux, V., Grosheny, D., Otero, O.,

467   Martineau, F., Fourel, F., Balter, V. and Reynard, B. (2012) Carbon and

468   oxygen isotope fractionations between aragonite and calcite of shells

469   from modern molluscs. *Chemical Geology*, **332-333**, 92-101.

470   Lee, M.R., Hodson, M.E. and Langworthy, G.N. (2008) Earthworms

471   produce granules of intricately zoned calcite. *Geology*, **36**, 943-946.

472   McConnaughey, T (1989) 13C and 18O isotopic disequilibrium in

473   biological carbonates II: *In vitro* simulation of kinetic isotope effects.

474   *Geochimica et Cosmochimica Acta*, **53**, 163-171.

475   Nielsen, L.C., DePaolo, D.J., De Yoreo, J.J. (2012) Self-consistent ion-by-

476   ion growth model for kinetic isotopic fractionation during calcite

477   precipitation. *Geochimica et Cosmochimica Acta*, **86**, 166–181.

478   Paul, D. and Skrzypek, G. (2007) Assessment of carbonate-phosphoric

479   acid analytical technique performed using gasbench ii in continuous

480   flow isotope ratio mass spectrometry. *International Journal of Mass*

481   *Spectrometry*, **262**, 180-186.

482   Politi, Y., Metzler, R. A., Abrecht, M., Gilbert, B., Wilt, F. H., Sagi, I., Addadi,

483   L., Weiner, S. and Gilbert, P. U. P. A. (2008) Transformation

484   mechanism of amorphous calcium carbonate into calcite in sea

485   urchin larval spicule. *Proceedings of the National Academy of Sciences*.

486   **105**, 17362-17366.

487   Pontoni, D., Bolze, J., Dingenouts, N., Narayanan, T. and Ballauff, M.

488   (2003) Crystallization of calcium carbonate observed in-situ by

489   combined small- and wide-angle X-ray scattering. *Journal of Physical*

490   *Chemistry B*, **107**, 5123-5125.

491   Radha, A.V., Forbes, T.Z., Killian, C.E., Gilbert, P.U.P.A. and Navrotsky, A.

492   (2010) Transformation and crystallization energetics of synthetic

493   and biogenic amorphous calcium carbonate. *Proceedings of the*

494   *National Academy of Sciences*, **107**, 16438-16443.

495   Rodriguez-Blanco, J.D., Shaw, S. and Benning, L.G. (2011) The kinetics

496   and mechanisms of amorphous calcium carbonate (acc)

497   crystallization to calcite, via vaterite. *Nanoscale*, **3**, 265-271.

498   Rodriguez-Blanco, J.D., Shaw, S. and Benning, L.G. (2008) How to make

499   "stable" ACC: protocol and preliminary structural characterization.

500   *Mineralogical Magazine*, **72**, 283-286.

501   Rollion-Bard, C., Saulnier, S., Vigier, N., Schumacher, A., Chaussidon, M.,

502   Lécuyer, C. (2016) Variability in magnesium, carbon and oxygen

503   isotope compositions of brachiopod shells: Implications for

504   paleoceanographic studies. *Chemical Geology*, **423**, 49-60.

505   Romanek, C.S., Grossman, E.L. and Morse, J.W. (1992) Carbon isotopic

506   fractionation in synthetic aragonite and calcite: Effects of

507   temperature and precipitation rate. *Geochimica et Cosmochimica*

508   *Acta*, **56**, 419-430.

509   Spooner, P.T., Guo, W.F., Robinson, L.F., Thiagarajan, N., Hendry, K.R.,

510   Rosenheim, B.E. and Meng, M.J. (2016) Clumped isotope composition

511   of cold-water corals: A role for vital effects? *Geochimica et*

512   *Cosmochimica Acta*, **179**, 123-141.

513   Stephens, C.J., Kim, Y.-Y., Evans, S.D., Meldrum, F.C. and Christenson, H.K.

514   (2011) Early stages of crystallization of calcium carbonate revealed

515   in picoliter droplets. *Journal of the American Chemical Society*, **133**,

516   5210-5213.

517   Szaran, J. (1997) Achievement of carbon isotope equilibrium in the

518   system HCO_3^- (solution)-CO_2(gas). *Chemical Geology*, **142**, 79-86.

519   Versteegh, E.A.A., Black, S. and Hodson, M.E. (2014) Environmental

520   controls on the production of calcium carbonate by earthworms. *Soil

521   Biology and Biochemistry*, **70**, 159-161.

522   Versteegh, E.A.A., Black, S., Canti, M.G. and Hodson, M.E. (2013)

523   Earthworm secreted calcium carbonate – a new palaeothermometer?

524   *Geochimica et Cosmochimica Acta*, **123**, 351-357.

525   Voigt, O. (1933) Die funktion der regenwurm-kalkdrüsen. *Zoologische

526   Jahrbücher: Abteilung für allgemeine Zoologie und Physiologie der

527   Tiere*, **52**, 677-708.

528   Watson, B.E. (2004) A conceptual model for near-surface kinetic

529   controls on the trace element and stable isotope composition of

530   abiogenic calcite crystals. *Geochimica et Cosmochimica Acta*, **68**,

531   1473–1488.

532   Wehrmeister, U., Jacob, D.E., Soldati, A.L., Loges, N., Häger, T. and

533   Hofmeister, W. (2011) Amorphous, nanocrystalline and crystalline

534   calcium carbonates in biological materials. *Journal of Raman*

535   *Spectroscopy*, **42**, 926-935.

536 Weiner S. and Addadi L. (2011) Crystallization pathways in

537   biomineralization. *Annual Reviews of Material Research*, **41**, 21–

538   40.

539 Zhang, J., Quay, P.D. and Wilbur, D.O. (1995) Carbon isotope

540   fractionation during gas-water exchange and dissolution of CO_2.

541   *Geochimica et Cosmochimica Acta*, **59**, 107-114.

542

Earthworms produce granules of calcium carbonate that form from an amorphous calcium carbonate suspension

The microspherulites of amorphous calcium carbonate coalesce and recrystallize

Fractionation of C isotopes occurs as the ACC recrystallizes with $\varepsilon_{calcite-ACC}$ = -1.20 $\pm$ 0.52%

This is consistent with a dissolution-reprecipitation pathway rather than solid state rearrangement

This may be important for the interpretation of $CaCO_3$-based C isotope environmental proxies.