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Heterogeneity of C and O stable isotope compositions among closely-spaced samples along layers at crests of stalagmites, and its implications for paleoenvironmental research

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Abstract: Modern carbon and oxygen stable-isotope analysis of stalagmites uses very small samples and thus allows high-resolution time-series sampling along vertical tracks less than 1 mm wide. However, this modern small-sample approach has generally not asked if heterogeneity of stable-isotope compositions along a layer at the crest of a stalagmite is sufficient that sampling along an alternate vertical track as little as 1 mm away from the track used might yield different compositions and trends. This study's eight-fold sampling along layers at their crests in ten stalagmites reveals that, in some stalagmites, variation in $\delta^{13}C$ and/or $\delta^{18}O$ between laterally-adjacent samples less than 1mm apart exceeds by many times the 95% confidence interval indicated by eight-fold analysis of a single well-mixed powder analyzed in the same run as the crestal samples. These results call into question the significance of excursions defined by single samples in high-resolution studies of paleo-environmental histories and suggest that replicate sampling is required to confirm the reality of those apparent excursions and events.

Keywords: Speleothems; caves, isotopes; paleoclimatology; paleoenvironments; homogeneity

INTRODUCTION

Since the time of Moore (1956), speleothems (largely stalagmites) have been interpreted as records of past climate, and soon time series of stable isotope data from stalagmites were used as paleoclimatological records (e.g., Gascoyne, 1992; Goede, 1994) in papers now numbering more than a thousand (Demény et al., 2024). Across that time, the size of the sample of $CaCO_3$ needed to generate a pair of $\delta^{13}C$ and $\delta^{18}O$ values has diminished greatly, from 5-10 mg down to 50-100 micrograms (Skrzypek and Paul, 2006; Paul and Skrzypek, 2007), and thus by a factor of 100. One of the previous large samples typically required collection of $CaCO_3$ powder from *a trench a few millimeters long drilled along a given lay*er (Fig. 1A), but the new small sample size allows analysis of a sample extracted with a dental drill bit from *a single circular hole roughly 0.6 mm in diameter* (Fig. 1B). The much smaller samples of the new method allow sampling along a much narrower vertical track (e.g., Series B_1 in Fig. 1B) that might have as readily been drilled along a track less than a millimeter to the right or left (e.g., Series B_2 in Fig. 1B).

In the older mode of sampling for larger amounts of power, drilling or milling a trench along a layer at the crest of a stalagmite layer could seemingly be trusted to mix and thus eliminate any heterogeneity of $\delta^{13}C$ or $\delta^{18}O$ along the layer's crest. However, the newer mode of sampling for a much smaller amount of powder at one point along the crest performs no such large-scale (>1 mm) homogenization. The newer mode of sampling (e.g., Series B_1 in Fig. 1B) thus presents the rarely-asked but significant question of whether an adjacent, typically-undrilled, series of small samples (e.g., Series B_2 in Fig. 1B) might have given different results. To test this troubling hypothesis that at least some values of $\delta^{13}C$ or $\delta^{18}O$ in a one vertical transect might differ from those in a parallel vertical transect less than a seemingly irrelevant millimeter away, this paper reports $\delta^{13}C$ or $\delta^{18}O$ from samples spaced less than 1 mm apart along layers in ten stalagmites from around the world.

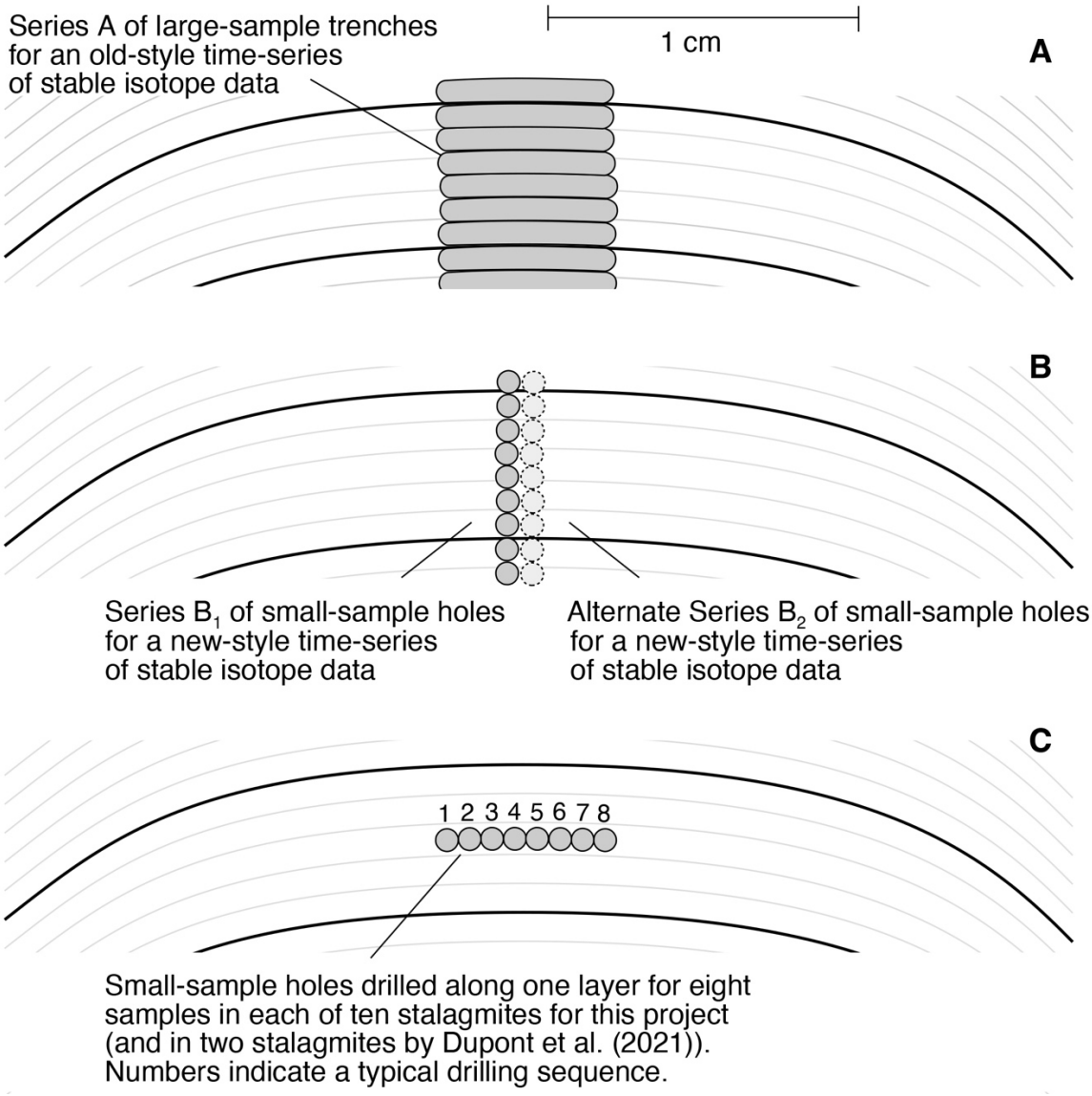

Fig. 1. A. Trenches of the sort historically used to generate samples of 5-10 mg of $CaCO_3$ powder for stable isotope analysis. B. Holes of the sort presently used to generate samples of 50-100 micrograms of $CaCO_3$ powder for stable isotope analysis. Of the two columns of holes, one set (B_1) represents a singles series customarily drilled to obtain C and O stable isotope data for paleoclimatological analysis, and the other set (B_2) represents a series rarely if ever drilled to determine along-layer variability of stable isotope composition at the crest of the stalagmite. C. Sampling plan to examine along-layer variability for this project, as shown in Figure 2.

METHODS

Ten stalagmites were selected for determination of $\delta^{13}C$ or $\delta^{18}O$ for this project (Table 1); they are shown in their entirety in Supplementary Figure S1. Criteria for selection included well-defined layering and a layer crest sufficiently broad that a span of 1 cm was entirely on the crest rather than on the sloping flanks. To avoid geographic bias, the ten stalagmites were from ten different nations from four different continents. All the stalagmites had been collected for other projects.

Table 1. Origin of stalagmites analyzed

Stalagmite	Nation	Region	Cave	Relevant publication(s)
BDS1	Botswana	Ngamiland	Drotsky's Cave	Railsback et al. (1994, 1999)
BZJC12	Belize	Cayo	Jose Cueva Cave	
CYUK1	Cyprus	Unknown	Unknown	
DH5	India	Uttarakhand	Dharamjali Cave	
EO1	Mexico	Oaxaca	Unknown	
ESP01	Spain	Galicia	Cova Paleira	Railsback et al. (2017)
MA3	Madagascar	Mitsinjo	Anjohibe Cave	Voarintsoa et al. (2017)
MH1	Namibia	Kunene	Mooihoek Cave	Wang (2016)
US-INIC9	United States	Indiana	Indiana Caverns	Akers (2016)
Wudu	China	Gansu	Wanxiang Cave	Railsback et al. (2014)

In each of the ten stalagmites, eight samples (each of 50-100 micrograms) were extracted along a single layer (Fig. 1C). The samples were extracted using SS Whyte HP-1/4 tungsten carbide dental burs, the drilling ends of which have a diameter of roughly 0.5 mm. The greatest span of sampling, from the left side of the leftmost hole to the right side of the rightmost hole, was 5.7 mm, a slightly smaller span than that in analogous sampling by Dupont et al. (2021). The collective lateral span of the eight samples was thus less than the breadth of a typical water droplet after falling onto a solid surface, satisfying the goal that there have been no difference in isotopic fractionation in the eight samples. The holes were approximately 0.5 mm deep. Examples are shown in Figure 2.

Each stalagmite's samples were analyzed as a group, so that all eight of the samples of Stalagmite BDS1 were analyzed, and then all eight of the samples of Stalagmite BZJC12, and so on. For each of the ten stalagmites, the samples were analyzed in the order of their position along the layer so that, for example, the samples of Stalagmite BDS1 (Fig. 2B) were analyzed in

the order BDS1-1, BDS1-2, BDS1-3, etc. The purpose of these specifications was to minimize any difference in results for adjacent samples that could have been caused by drift of the mass spectrometer during the analysis of the entire suite of 88 powders. In addition to the 8 closely-spaced but distinct samples from each of ten stalagmites described above, eight splits of a well-

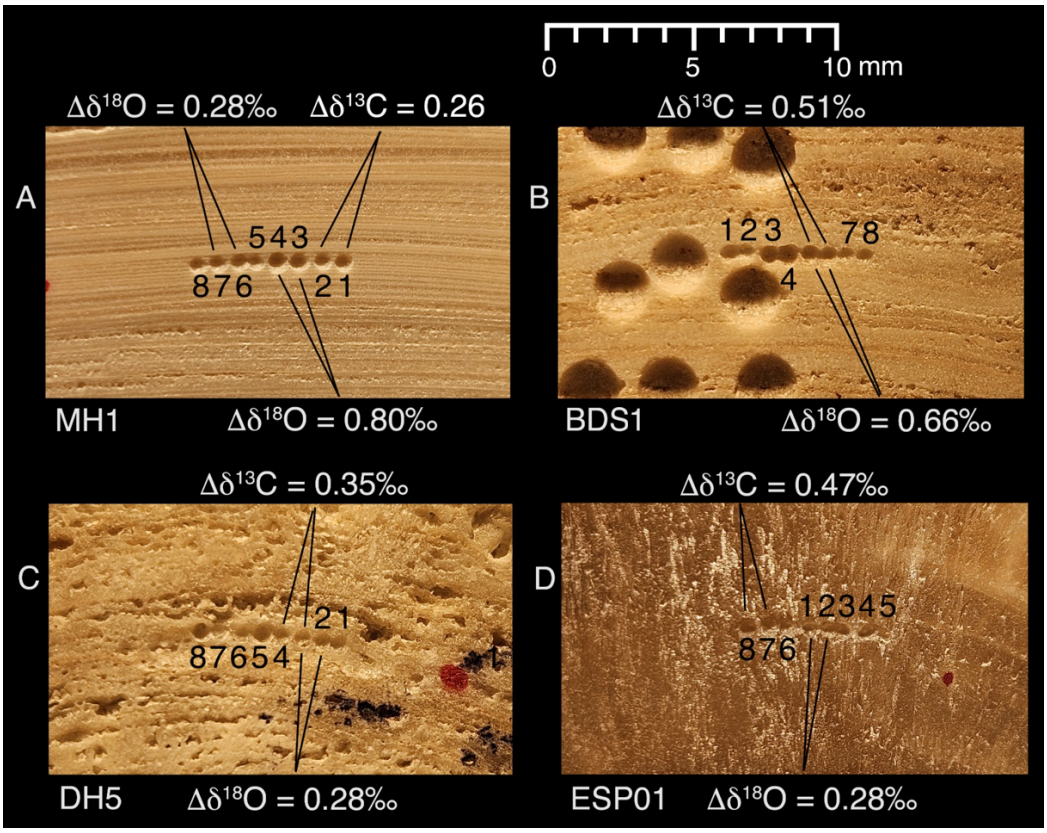

Fig. 2. Examples of drilling performed and data collected for this study. Numbers from 1 to 8 indicate order of drilling and order of analysis. Each pair of black lines points to adjacent holes for two consecutively-analyzed samples from which the difference in carbon isotopic composition ($\Delta\delta^{13}C$) and/or difference in oxygen isotopic composition ($\Delta\delta^{18}O$) was large. Note that, although not all holes are identical in size, those joined by pairs of black lines are essentially identical in size but nonetheless have considerable difference in $\delta^{13}C$ and/or $\delta^{18}O$, so that subtle differences of drilling style do not explain the observed large differences in isotopic composition. In B, the large holes in Stalagmite BDS1 were from a much earlier sampling program.

mixed powder of spelean aragonite (CCStd1) were analyzed for determination of $\delta^{13}C$ or $\delta^{18}O$ as a control group. The samples of CCStd1 were analyzed in sequence as a group between the groups of samples from the ten stalagmites, so that the mass-spectrometer's performance in analyzing splits of CCStd1 would be as representative of its performance with the stalagmite samples as possible, rather than reflecting the machine's performance before or after the stalagmite samples were analyzed.

The samples described above were analyzed using a Thermo Scientific Delta V Plus mass spectrometer paired with a GasBench preparation system in the Alabama Stable Isotope Laboratory (ASIL) of the Department of Geology of the University of Alabama using the methods of Skrzypek and Paul (2006), Paul and Skrzypek (2007), and Lambert and Aharon (2011). Calcite reference material IAEA-603 ($\delta^{13}C$ = +2.46, $\delta^{18}O$ = –2.37% relative to VPDB) was employed as an internal standard. There were no significant time trends or beam size relationships for standards, both for C and O. Four samples yielded no usable data, dictating use of the expression "seven or eight" in the results presented below. The data are reported here relative to the Vienna PeeDee Belemnite (VPDB) standard.

RESULTS

Ranges of values of $\delta^{13}C$ and $\delta^{18}O$

For all ten stalagmites, the ranges of values of $\delta^{13}C$ and $\delta^{18}O$ from seven or eight closely spaced crestal samples from one layer (Fig. 1C) were greater than 0.17‰ and greater than the ranges measured in a well-mixed powder of stalagmite $CaCO_3$ from CCStd1 (Fig. 3). In five of the ten stalagmites, ranges of values of $\delta^{13}C$ and $\delta^{18}O$ were both greater than 0.5‰, and in one case (Stalagmite MH1) the range of $\delta^{18}O$ values among the eight samples exceeded 0.8‰. These ranges were not the result of instrumental variability: eight samples of a well-mixed powder of stalagmite $CaCO_3$ (CCStd1) were analyzed on the same machine during the same run, and they yielded smaller ranges of $\delta^{13}C$ and $\delta^{18}O$ than any of the sets of samples taken along stalagmite layers. Further evidence that the ranges were not a result of instrumental variability is that the set of eight crestal powders with the least variation, that of Stalagmite US-INIC9, was run immediately after the set of eight crestal powders with the greatest variation, that of Stalagmite MH1 (Table 2).

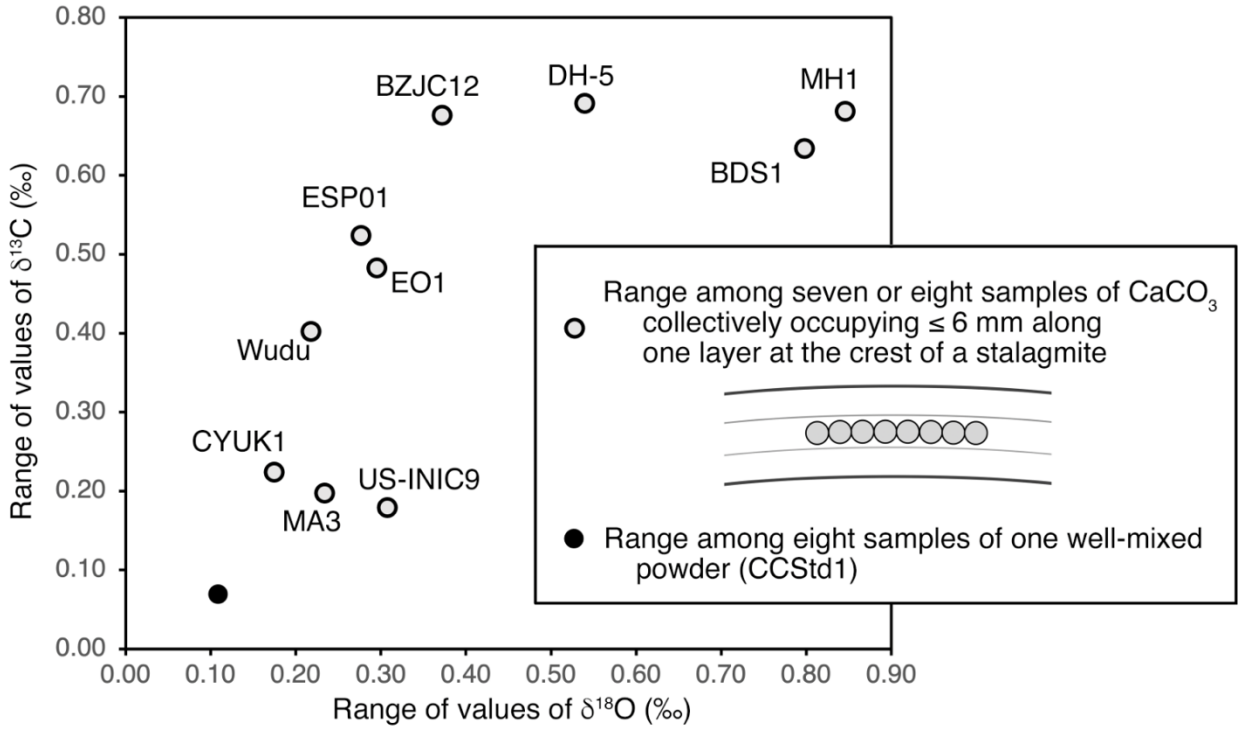

Fig. 3. Plot of ranges of C and O isotopic compositions of either seven or eight samples of $CaCO_3$ from along one layer of a stalagmite (gray-filled symbols) and of the range of C and O isotopic compositions of one well-mixed powder of stalagmite $CaCO_3$ (black-filled symbol). The data are shown in Table 2.

Differences between adjacent samples

The previous paragraph reports results in terms of *groups* of samples from stalagmite layers; equally striking are differences in $\delta^{13}C$ and $\delta^{18}O$ between *adjacent* samples in the eight-member sets of samples drilled along stalagmite layers (Figs. 1C and 2). In this case, among eight samples lettered in order laterally as A-B-C-D-E-F-G-H, the differences considered are A-B, B-C, C-D, D-E, E-F, F-G, and G-H, but not A-C, A-D, B-D. etc. In all cases the differences reported are therefore between samples less than 1 mm apart. Four samples yielded no usable data, so that rather than the expected 70 differences expected from eight-sample suites from ten stalagmites, only 63 differences were generated.

Table 2. Stable isotope data in order of analysis

Sample ID	$\delta^{13}C$ (‰ VPDB)	$\delta^{18}O$	Adjacent difference[1] $\Delta\delta^{13}C$	$\Delta\delta^{18}O$
Adjacent ~75µg samples of Stalagmite BDS1:				
BDS97014-1	-8.69	-8.58	-0.14	-0.17
BDS97014-2	-8.55	-8.41	-0.50	-0.53
BDS97014-3	-8.06	-7.88	0.20	0.23
BDS97014-4	-8.25	-8.11	0.00	-0.08
BDS97014-5	-8.25	-8.03	-0.16	-0.25
BDS97014-6	-8.10	-7.78	0.51	0.66
BDS97014-7	-8.60	-8.44	-0.15	-0.19
BDS97014-8	-8.46	-8.25		
Adjacent ~75µg samples of Stalagmite BZJC12:				
BZJC12-1	-7.98	-4.37	0.10	0.01
BZJC12-2	-8.09	-4.38		
BZJC12-3 All peaks below minimum of 1000 mV				
BZJC12-4	-7.89	-4.26	-0.09	-0.11
BZJC12-5	-7.80	-4.16	0.24	0.24
BZJC12-6	-8.04	-4.40	0.25	0.13
BZJC12-7	-8.29	-4.53	0.19	-0.15
BZJC12-8	-8.48	-4.37		
Splits of well-mixed powder of CCStd1:				
CCStd1-9	-5.72	-8.05	0.07	0.09
CCStd1-10	-5.79	-8.14	-0.01	-0.05
CCStd1-11	-5.78	-8.09	-0.05	-0.01
CCStd1-12	-5.73	-8.08	0.02	-0.03
CCStd1-13	-5.75	-8.05	0.01	-0.01
CCStd1-14	-5.75	-8.04	0.00	0.11
CCStd1-15	-5.76	-8.15	0.01	-0.05
CCStd1-16	-5.76	-8.10		
Adjacent ~75µg samples of Stalagmite CYUK1-1:				
CYUK1-1	-7.28	-6.24	0.06	0.01
CYUK1-2	-7.34	-6.24	-0.20	-0.08
CYUK1-3	-7.15	-6.16	0.18	0.14
CYUK1-4	-7.32	-6.31		
CYUK1-5 All peaks below minimum of 1000 mV				
CYUK1-6	-7.33	-6.30	0.04	0.00
CYUK1-7	-7.37	-6.29	-0.09	0.04
CYUK1-8	-7.28	-6.34		
Adjacent ~75µg samples of Stalagmite DH5-1:				
DH5-1	-8.54	-7.57	0.02	0.15
DH5-2	-8.57	-7.72	-0.34	-0.28
DH5-3	-8.23	-7.44	-0.35	-0.26
DH5-4	-7.88	-7.19	0.24	0.12
DH5-5	-8.11	-7.31	0.36	0.35
DH5-6	-8.47	-7.65	-0.40	-0.32
DH5-7	-8.07	-7.34		
DH5-8	No sample gas (clog in needle?)			
Adjacent ~75µg samples of Stalagmite E01:				
EO1-1	-9.20	-9.39	0.11	-0.01
EO1-2	-9.31	-9.38	-0.12	-0.12
EO1-3	-9.19	-9.26	-0.12	0.02
EO1-4	-9.07	-9.28	0.27	0.14
EO1-5	-9.34	-9.41	0.12	0.11
EO1-6	-9.46	-9.52	0.09	0.03
EO1-7	-9.55	-9.55	-0.08	-0.17
EO1-8	-9.47	-9.38		
Adjacent ~75µg samples of Stalagmite ESP01:				
ESP01-1	-5.44	-5.96	-0.11	-0.28
ESP01-2	-5.32	-5.68	0.17	0.03
ESP01-3	-5.49	-5.71	-0.11	-0.03
ESP01-4	-5.39	-5.68	0.12	0.14
ESP01-5	-5.50	-5.82	Not adjacent[2]	
ESP01-6	-5.43	-5.84	-0.06	0.03
ESP01-7	-5.38	-5.87	0.47	0.08
ESP01-8	-5.85	-5.95		
Adjacent ~75µg samples of Stalagmite MA3:				
MA3-305-1	-9.31	-4.50	-0.10	-0.03
MA3-305-2	-9.21	-4.47		
MA3-305-3	No sample gas (clog in needle?)			
MA3-305-4	-9.29	-4.56	-0.01	-0.10
MA3-305-5	-9.28	-4.47	0.02	-0.02
MA3-305-6	-9.30	-4.45	0.11	-0.03
MA3-305-7	-9.41	-4.41	-0.12	-0.08
MA3-305-8	-9.29	-4.33		
Adjacent ~75µg samples of Stalagmite MH1:				
MH1-1	2.64	-1.89	0.01	-0.33
MH1-2	2.62	-1.57	0.12	0.28
MH1-3	2.51	-1.85	0.26	-0.80
MH1-4	2.24	-1.05	-0.10	0.54
MH1-5	2.34	-1.59	0.23	-0.04
MH1-6	2.12	-1.55	0.16	0.28
MH1-7	1.96	-1.83	-0.17	-0.01
MH1-8	2.13	-1.82		
Adjacent ~75µg samples of Stalagmite US-INIC9:				
US-INIC9-1	-8.17	-5.22	-0.04	0.04
US-INIC9-2	-8.13	-5.26	0.11	-0.05
US-INIC9-3	-8.24	-5.22	0.04	-0.09
US-INIC9-4	-8.28	-5.13	0.00	0.02
US-INIC9-5	-8.28	-5.15	0.01	0.28
US-INIC9-6	-8.29	-5.44	-0.07	-0.20
US-INIC9-7	-8.22	-5.24	0.09	0.01
US-INIC9-8	-8.31	-5.26		
Adjacent ~75µg samples of Stalagmite Wudu:				
Wudu43-1	-4.16	-6.23	0.07	-0.01
Wudu43-2	-4.23	-6.22	-0.02	-0.13
Wudu43-3	-4.21	-6.09	0.03	0.03
Wudu43-4	-4.24	-6.12	0.18	0.07
Wudu43-5	-4.42	-6.19	0.11	0.11
Wudu43-6	-4.53	-6.31	0.03	-0.17
Wudu43-7	-4.56	-6.14	-0.09	0.00
Wudu43-8	-4.47	-6.13		

[1] The value shown is the difference between that sample and the sample in the following line of the table. These values were used to construct Figure 4.

[2] The direction of drilling in Stalagmite ESP01 was reversed between Samples 5 and 6, so that those two samples were not adjacent (see Fig. 2D).

For both $\delta^{13}C$ and $\delta^{18}O$, most of the differences in $\delta^{13}C$ and $\delta^{18}O$ between adjacent samples are greater than 0.1‰ (Figs. 4C and 4D). For $\delta^{13}C$, 13 of the 63 differences between adjacent samples are greater than 0.2‰, and for $\delta^{18}O$, 16 of the 65 differences between adjacent samples are greater than 0.2‰. The most extreme cases are 0.51‰ for $\delta^{13}C$ (Fig. 2B) and 0.80 for $\delta^{18}O$ (Fig. 2A). These results contrast strongly with the data from well-mixed material CCStd-1, where the greatest difference between consecutive determinations of $\delta^{13}C$ is 0.07‰ and the greatest difference between consecutive determinations of $\delta^{18}O$ is 0.11‰ (Figs. 4A and 4B).

One might argue that the large differences in $\delta^{13}C$ and $\delta^{18}O$ between adjacent samples could be the result of disequilibrium fractionation (Hendy, 1971). However, the small (<0.6 mm) spacing between samples makes this process unlikely to be relevant, and there is no pattern of increasing $\delta^{18}O$ and/or $\delta^{13}C$ toward the flanks of the stalagmite (Table 2 and Supplementary Figure S2). Alternately, one might argue that the large differences in $\delta^{13}C$ and $\delta^{18}O$ between adjacent samples might result from small differences in size of the holes from which the samples come, with the larger of the two holes sampling material above and below the material sampled by the smaller hole. However, close examination shows that this is not the case, and that in fact many of the largest differences come from pairs of identical holes (Fig. 2), whereas differences between holes of slightly different size are commonly smaller.

DISCUSSION

The results from ten stalagmites reported above and illustrated in Figures 3 and 4 provide further evidence of isotopic heterogeneity along layers previously reported in two stalagmites by Dupont et al. (2021). In that study, differences in $\delta^{13}C$ between adjacent samples less than 1 mm apart were as great as 0.44‰ and as great as 0.26‰ for $\delta^{18}O$ (Figs. 4E and 4F). Similarly, in samples spaced 2 mm apart across a stalagmite with flat-topped layers, Tan et al. (2015) found a difference of 0.4‰ in $\delta^{13}C$ between adjacent samples and 0.5‰ in $\delta^{18}O$ between adjacent samples. Thus, although this study presents significantly more data than previous research, and it reports some larger ranges along layers and larger differences between neighboring samples, its

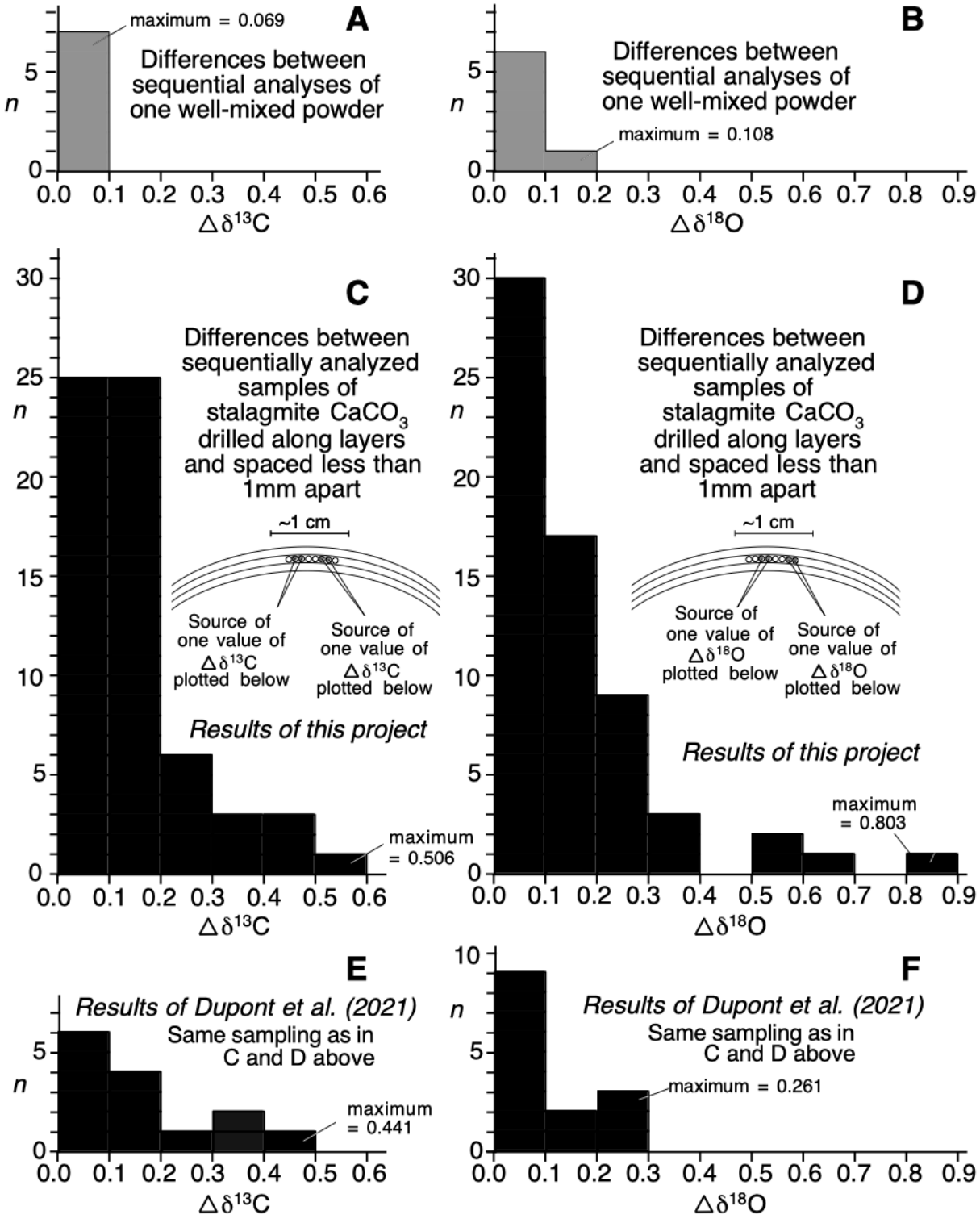

Fig. 4. Histograms showing differences in $\delta^{13}C$ and $\delta^{18}O$ between adjacent samples less than 1 mm apart along growth layers at crests of stalagmites. The data are given in Table 2.

findings only confirm a previously-reported if little-appreciated heterogeneity of isotope composition among closely-spaced samples along stalagmite layers at stalagmite crests.

The heterogeneity of isotopic compositions between adjacent samples along stalagmite layers reported here is also not unique among sedimentary materials. Theiling et al. (2007) demonstrated isotopic heterogeneity along limestone layers, with greatest heterogeneity near surfaces of subaerial exposure but with ranges exceeding 1‰ along layers away from those surfaces. Dupont et al. (2021) sampled a variety of non-marine carbonate materials and found ranges of $\delta^{13}C$ and $\delta^{18}O$ as great as 5‰ along layers or crusts. Those results, while not directly relevant to stalagmites, demonstrate that isotopic heterogeneity along layers in Earth-surface carbonates far exceeding analytical uncertainty is not surprising and must be considered in interpreting isotopic records. In that greater context, this paper demonstrates, as did Dupont et al. (2021), that such heterogeneity can exist at sample spacings less than 1 mm.

CONCLUSIONS

Despite analytical precision of 0.1‰ or less, differences in $\delta^{13}C$ and $\delta^{18}O$ several times that much between single samples of $CaCO_3$ in a stalagmite's time series should not be taken as evidence of paleoenvironmental change. Instead, an excursion of as much as 0.8‰ that is so short-lived that it is represented by one sample in a time series must be confirmed by time-equivalent adjacent samples demonstrating a change in mean layer composition in the seemingly anomalous layer. Demonstration not only of the excursion but also of its short-lived nature as an event would additionally require analogous time-equivalent adjacent samples in the layers just above and below the inferred short-lived excursion.

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Supplementary information for

Heterogeneity of C and O stable isotope compositions among closely-spaced samples along layers at crests of stalagmites, and its implications for paleoenvironmental research

by L. Bruce Railsback

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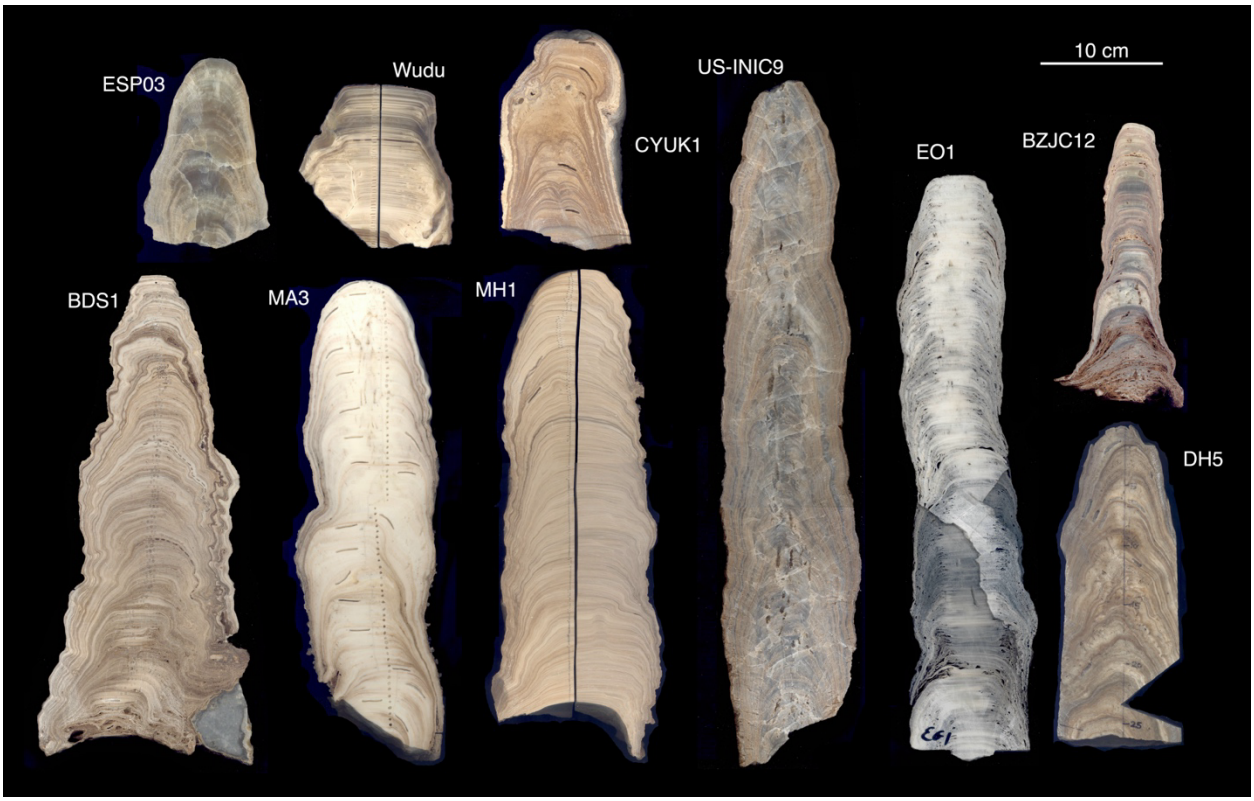
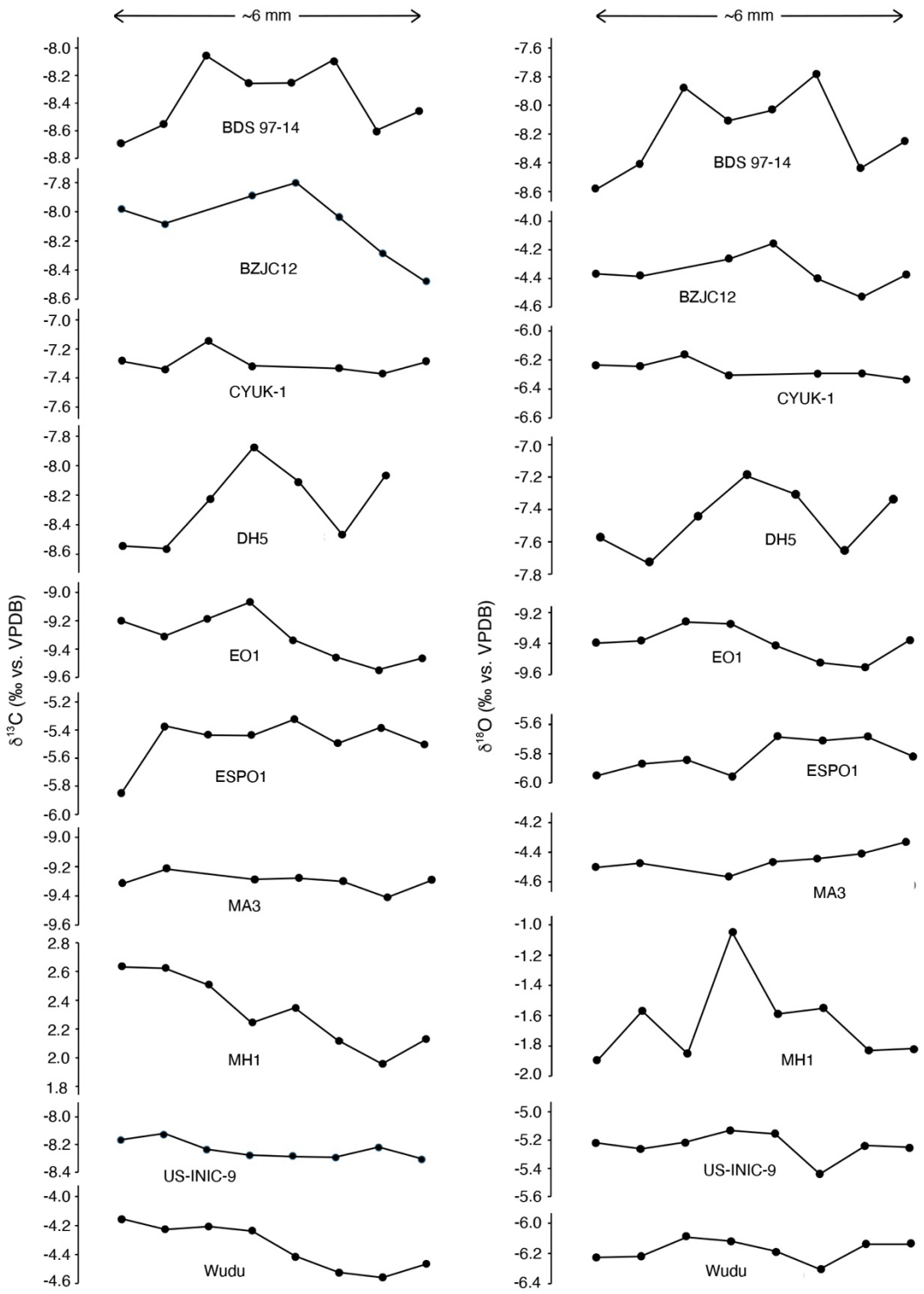


Supplementary Figure 1: Image of the ten stalagmites sampled for this project.



Supplementary Figure 2: Plots of isotopic compositions of samples against the positions of those samples.