EFFECTS OF SAMPLE PRE-TREATMENT ON THE $\delta^{13}\text{C}$ AND $\delta^{18}\text{O}$ VALUES OF LIVING BENTHIC FORAMINIFERA

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Abstract

Stable carbon and oxygen isotope ratios ($\delta^{13}\text{C}$ and $\delta^{18}\text{O}$) of the epibenthic foraminiferan *Elphidium macellum* var. *aculeatum* were measured and compared both in raw aliquots and after various pre-analytical treatments. We tested the effects of chemical cleaning procedures (oxygen plasma ashing, NaClO and H_2O_2) and physical (ultrasounds) aimed at removing allochthonous particles. We also assessed the effects of using preservatives (formaldehyde and ethanol) and stains (Bengal Rose and Sudan Black B) prior to $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ analyses. Although the effects were generally small, three of the pre-treatments had a significant effect on $\delta^{13}\text{C}$ or $\delta^{18}\text{O}$ values. The use of Sudan Black B for vital identification caused a significant enrichment in the ^{13}C of the samples (0.24%). In addition, sample oxidation with hydrogen peroxide and ultrasonic cleaning each led to a slight decrease in $\delta^{18}\text{O}$ values (-0.18‰, in both cases). Our results demonstrate that Sudan Black B should be avoided prior to isotopic analysis. Bengal Rose showed no significant effect and should be used instead for staining. Because palaeoclimatic studies are based in small isotopic shifts, we discourage the pre-analysis use of unnecessary harsh cleaning treatments of foraminifera shell samples that may lead to isotopic fractionation and hence confound ecological interpretation.

1. Introduction

The isotopic composition of foraminifera shells is widely used in palaeoreconstructions (e.g., palaeoecology, palaeontology, palaeoclimatology) to make inferences about the physical and biological paleo-environments where the organisms grew. Foraminifera calcite tests are composed of pure calcite (>99%) with a relatively simple mineralogical structure (Milliman, 1974). An early study by Walton (1952) described the presence of potential sources of $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ variability in carbonate foraminifera shells associated with the presence of chitin, detritic minerals, organic rests, etc. The impurities introduced into a mass spectrometer may alter measured $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ values of foraminifer's carbonates (e.g., Epstein et al., 1951; Charef and Sheppard, 1984).

Over the past few decades, most geologists have used several pre-treatments prior to isotopic analyses to eliminate allochthonous organic and inorganic particles attached to the sample shells. Typical organic matter removal techniques include oxidizing pre-treatments such as NaOCl and H_2O_2 bleaching and oxygen plasma ashing. While some attention has already been paid to the effect of oxidizing methods on the $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ values of planktonic foraminifera (e.g., Duplessy et al., 1970; Sarkar et al., 1990; Waelbroeck et al., 2005), the effects of most of these pre-treatments on living benthic foraminifera remain unknown. In addition, the isotope composition of benthic foraminifera is characterized by higher variability than the composition of planktonic foraminifera due to their environment of calcification (Waelbroeck et al., 2005). A good pre-treatment should be effective in the removal of exogenous contaminants attached to the shell while leaving the isotopic composition of the calcite unaltered. Studies of palaeoclimatology rely heavily on accurate detection of small isotopic shifts: e.g., a 1‰ decrease in $\delta^{18}\text{O}$ may result in 4°C increase in paleo-temperature estimates (Shackleton, 1974), and, consequently, isotopic shifts derived from pre-treatments may confound a correct interpretation. Isotopic shifts could also be caused by other variables: (1) poor analytical reproducibility, (2) inadequate sample preparation techniques (Waelbroeck et al., 2005), (3) vital effects (Urey, 1947; Wefer and Berger, 1991) due to

inter-genotype variability (Hecht & Savin, 1972) or disequilibrium precipitation (e.g., Duplessy et al., 1970), or (4) different shell morphologies (e.g., Duplessy, 1978; Berger et al., 1978).

The number of isotopic studies based on living foraminifera has substantially increased in recent years (e.g., Filipsson et al., 2004). The success of these investigations depends on accurate techniques for discriminating between living and non-living forms. In the great majority of studies, foraminifera are stained for vital determination prior to isotopic analysis (e.g., Mackensen et al., 2000; Fontanier et al., 2006). However, the effects of staining on $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ foraminifera calcite are not fully understood. Different kinds of preservatives (e.g., formalin, ethanol) are also used when samples cannot be processed immediately after sampling. Their effects on the isotopic signatures are likewise poorly known.

2. Methods

In order to assess the effect of pre-analytical treatments on $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ analyses, individuals of *Elphidium macellum* var. *aculeatum* from *Posidonia oceanica* leaves were collected in June 2006 using SCUBA equipment at 5m depth in a meadow growing in Portlligat Bay, Girona, Spain (42° 17' 32.3'' N/ 3° 17' 23.6'' E, Western Mediterranean). Immediately after sampling, the leaves were gently rubbed by hand in 63 μm -sieved seawater. The resulting material was filtered through a 125 μm sieve and deposited into sealed buckets. Samples were transported in an aerated cool box to the laboratory within two hours after sampling. Once in the laboratory, samples were kept at 14°C to minimize bacterial growth and oxygen depletion (Arnold, 1954).

Living specimens, i.e., those with colored protoplasm and reticulopodial activity, were selected under an inverted microscope using soft 4-0 sable brushes. Juvenile individuals between 450-550 μm and 350-430 μm (maximum and minimum diameter, respectively) were selected to minimize size and vital effects on isotopic values (e.g., Savin and Douglas, 1973; Boltovskoy and Wright,

1976; Berger et al., 1978). Each pooled sample consisted of four specimens, an adequate amount
76 of carbon and oxygen for one isotopic analysis (i.e., >10 µg C and >30 µg O).

All replicates (control and treated samples) were rinsed with distilled water by vortex agitation.

78 Pre-analytical treatments were applied to three or four pooled replicates (Table 1). The average
isotopic composition of six untreated replicates was used as a control value. Twelve pre-analytical
80 treatments were applied: (1) a 4% formaldehyde solution; (2) a 70% ethanol solution; (3) Bengal
Rose ($C_{20}H_2Cl_4I_4Na_2O_5$) in saturated solution, 1g/l (Walton, 1952); (4) Sudan Black B ($C_{29}H_{24}N_6$)
82 in 70% ethanol solution (Walker et al., 1974); (5) a combination of Bengal Rose and formaldehyde
pre-treatments, (6) a combination of Bengal Rose and ethanol pre-treatments; (7) plasma ashing,
84 performed in a Polaron plasma barrel etcher PT 7150 for 24 h; (8) a 10% NaClO solution bath for
10 min at 20°C; (9) a 30% H_2O_2 solution bath for 10 min at 20°C; (10) 10 seconds of a methanol
86 ultrasonic bath at 20°C; (11) a combination of ultrasonic bath and NaClO pre-treatments; and (12)
a combination of ultrasonic bath and H_2O_2 pre-treatments. The effect of preservatives was tested
88 after 50 days. Samples were oven-dried at 60°C for at least 72 h and transferred to acid-washed
vials. The stable isotopic composition was determined using a Thermo-Finnigan MAT 252 mass
90 spectrometer coupled to an automated carbonate preparation device (Scientific-Technical Services,
University of Barcelona). Isotopic results were calibrated to the Vienna PeeDee Belemnite
92 (VPDB) standard via the NBS19 standard. The analytical reproducibility of the laboratory standard
was better than $\pm 0.07\text{‰}$ and $\pm 0.03\text{‰}$ (SD, $n = 14$) for $\delta^{18}O$ and $\delta^{13}C$, respectively. All data were
94 checked for normality (Kolmogorov-Smirnov test) and for variance homogeneity (Levene's test).
We used ANOVA and post-hoc Tukey HSD tests to assess the null hypothesis of differences
96 between treated and untreated samples (rejected when $p \leq 0.05$). By selecting individuals of
specific sizes and correctly identifying vital activity, we achieved low isotopic variability between
98 replicates both for $\delta^{13}C$ and $\delta^{18}O$ values (mean S.E.M. = 0.05‰ and 0.04‰, respectively).

3. Results and Discussion

Only 3 of the 12 treatments applied yielded significant differences in $\delta^{13}\text{C}$ or $\delta^{18}\text{O}$ values with respect to the control aliquots (Table 1, Figure 1). The $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ values ranged from -0.29 to 0.03‰ and from 0.33 to 0.63‰, respectively. Post-hoc tests showed that staining with Sudan Black B caused a significant ^{13}C enrichment effect (0.24‰, $P < 0.05$), while the mean $\delta^{18}\text{O}$ values of the samples oxidized with hydrogen peroxide as well as the ultrasonic-cleaned samples were slightly, but significantly lower than those of the control aliquots (0.18‰, $P < 0.05$, in both cases). The advantages and disadvantages of using Bengal Rose or Sudan Black B stains for the identification of live foraminifera have been the subject of a long-standing controversy (Boltovskoy and Lena, 1970; Walker et al., 1974). Our results show that Bengal Rose is the better choice for the identification of live foraminifera. Usually, samples cannot be processed immediately after sampling, and, therefore, they often need to be preserved. We have shown that both ethanol and formalin preservatives can be used prior to $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ analyses. Formaldehyde, however, has been reported to alter carbonate isotopic signatures (Bé and Anderson, 1976; Ganssen, 1981). In general, the use of preservatives should be avoided in isotopic studies based on living foraminifera due to potential effects on isotopic values and despite the fact that processing the sample right after sampling makes it easier to identify living foraminifera, thanks to the movement and color of the remaining protoplasm. Several studies have addressed the usefulness and effectiveness of organic matter removal methods (Bellefleur, 1976; Conger et al., 1977; Gaffey and Bronnimann, 1993; Ito, 2001). In addition, large uncertainties remain in regard to the effects of such methods on $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ values (Duplessy et al., 1970; Hodgkinson, 1991; Sarkar et al., 1990). The decrease in shell $\delta^{18}\text{O}$ when pre-treated with H_2O_2 is consistent with Grottoli et al. (2005), who reported a decrease in the $\delta^{18}\text{O}$ of coral skeletal samples, and with Wierzbowski (2007), who reported a significant decrease in synthetic calcite $\delta^{18}\text{O}$ when pre-treated with H_2O_2 . Recently, it was demonstrated that the presence of organic

matter has no effect on oxygen and carbon isotope analyses as long as calcium carbonate decomposition is conducted with pure orthophosphoric acid. H₂O₂ treatment may, therefore, result in partial dissolution of calcium carbonates (Grottoli et al., 2005; Wierzbowski, 2007).

From our results, we also conclude that none of the pre-analytical treatments we assessed that were intended to remove organic matter attached to foraminifera shells are necessary prior to isotopic analyses. Hence, the slight isotopic shifts observed after ultrasonic cleaning may be a result of the elimination of inorganic contaminants, or it may be due to a partial dissolution of the skeleton. Ultrasonic agitation should be, in principle, discouraged as it seems to cause unknown isotopic fractionations and has not been proved as successful as initially expected (Stevens et al., 1960; Clark, 1973; Katz and Man, 1979). Considering that chemical and physical pre-analytical treatments are potential sources of alteration of isotopic composition, and that their effects may differ between species, a general recommendation should be to avoid the application of such treatments. This is consistent with recent recommendations for corals (Grottoli et al., 2005), ostracods (Keatings et al., 2006) and a range of carbonates (Wierzbowski, 2007). This study does not provide universal guidelines for all species of foraminifera or for different pre-treatment designs, and further tests should be done to evaluate whether these findings can be generalized. The lack of standard procedures for cleaning, staining and storing foraminiferan samples prior to isotopic analysis will likely preclude rigorous comparison of results across studies. In order to make all foraminiferan isotopic data comparable, a standard protocol should be developed and put into general use everywhere.

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References

- Arnold, Z.M., 1954. Field and laboratory techniques for the study of living foraminifera. In: Foraminifera (R.H. Headley and C.G. Adams, Eds), pp. 153-203. Academic Press, London.
- Bé, W.H., Anderson, O.R., 1976. Preservation of planktonic foraminifera and other calcareous plankton. In: Steedman, H. F. (Eds.), Zooplankton Fixation and Preservation. Paris: The Unesco Press, pp. 250-258.
- Bellefleur, S., 1976. Wall ultramicrostructure in the foraminifer *Cibicides floridanus* (Cushman). *Micropaleontology*, 22, 352-362.
- Berger, W.H., Killingley, J.S., Vincent, E., 1978. Stable isotopes in deep-sea carbonates. *Oceanologica Acta* 1, 203-316.
- Boltovskoy, E., Lena, H., 1970. On the decomposition of the protoplasm and the sinking velocity of the planktonic foraminifera. *Internationale Revue de gesamten Hydrobiologie und Hydrographie* 55, 797-804.
- Boltovskoy, E., Wright, R., 1976. Recent Foraminifera (second edition): Dr. W. Junk, The Hague, Netherlands, 515 p.
- Charef, A., Sheppard, S.M.F., 1984. Carbon and oxygen isotope analysis of calcite or dolomite associated with organic matter. *Isotope Geoscience* 2, 325-333.
- Clark, D.F., 1973. Effects of ultrasound on calcareous nannofossils. *Geology* 1, 61-62.

176 Conger, S.D., Green, H.W., Lipps, J.H., 1977. Test ultrastructure of some calcareous foraminifera.
 Journal of Foraminiferal Research 7, 278-296.

178 Duplessy, J.C., Lalou, C., Vinot, A.C., 1970. Differential isotopic fractionation in benthic
 foraminifera and paleotemperatures reassessed. Science 168, 250-251.

180 Duplessy, J.C., 1978. Isotope studies. In: Gribbin, J. (Eds.), Climatic Changes. Cambridge
 University Press, Cambridge, pp. 46-67.

182 Epstein, S., Buchsbaum, R., Lowenstam, H., Urey, H.C., 1951. Carbonate-water isotopic
 temperature scale. Geological Society of America bulletin 62, 417-425.

184 Filipsson, H.L., Nordberg, K., Gustafsson, M., 2004. Seasonal study of $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ in living
 (stained) benthic foraminifera from two Swedish fjords. Marine Micropaleontology 53, 159-172.

186 Fontanier, C., Mackensen, A., Jorissen, F.J., Anschutz, P., Licari, L., Griveaud, C., 2006. Stable
 oxygen and carbon isotopes of live benthic foraminifera from the Bay of Biscay: Microhabitat
 impact and seasonal variability. Marine micropaleontology 58, 159-183.

188 Gaffey, s.J., Bronnimann, C.E., 1993. Effects of bleaching on organic and mineral phases in
 biogenic carbonates. Journal of Sedimentary Petrology 63, 752-754.

190 Ganssen, G., 1981. Isotopic analysis of foraminifera shells: interference from chemical treatment.
 Palaeogeography, Palaeoclimatology, Palaeoecology 33, 271-276.

192 Grottoli, A.G., Rodrigues, L.J., Matthews, K.A., Palardy, J.E., Gibb, O.T., 2005. Pre-treatment
 effects on coral skeletal $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$. Chemical Geology 221, 225-242.

194 Hecht, A.D., Savin, S.M., 1972. Phenotypic variation and oxygen isotope ratios in Recent
 planktonic foraminifera. Journal of Foraminiferal Research 2, 55-67.

196 Hodgkinson, R.L., 1991. Microfossil processing: a damage report. Micropaleontology 37, 320-
 326.

198 Ito, E., 2001. Application of stable isotope techniques to inorganic and biogenic carbonates. In:
Last, W.M., Smol, J.P. (Eds.), *Tracking Environmental Change Using Lake Sediments*, vol. 2.
200 *Physical and Chemical Techniques*, Kluwer Academic Publishers, 351-371.

Katz, B.J., Man, E.H., 1979. Effect of ultrasonic cleaning on the amino acid geochemistry of
202 foraminifera tests. *Geochimica et Cosmochimica Acta* 43, 1567-1570.

Keatings, K.W., Holmes, J.A., Heaton, T.H.E., 2006. Effects of pre-treatment on ostracod valve
204 chemistry. *Chemical Geology* 235, 250-261.

Mackensen, A., Schumacher, S., Radke, J., Schmidt, D.N., 2000. Microhabitat preferences and
206 stable carbon isotopes of endobenthic foraminifera: clue to quantitative reconstruction of oceanic
new production? *Marine Micropaleontology* 40 (3), 233-258.

208 Milliman, J.D., 1974. Recent sedimentary carbonates. *Marine Carbonates*, Part 1, Springer-Verlag,
Berlin.

210 Sarkar, A., Ramesh, R., Bhattacharya, S.K., 1990. Effect of sample pretreatment and size fraction
on the $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ values of foraminifera in Arabian Sea sediments. *Terra nova* 2, 489-493.

212 Savin, S.M., Douglas, R.G., 1973. Stable isotope and magnesium geochemistry of recent
planktonic foraminifera from the south Pacific. *Geological Society of America bulletin* 84, 2327-
214 2342.

Shackleton, N.J., 1974. Attainment of isotopic equilibrium between ocean water and benthonic
216 foraminifera genus *Uvigerina*: isotopic changes in the ocean during the last glacial. *Les méthodes
quantitatives d'étude des variations du climat au cours du Pleistocène*, Gif-sur-Ivette. Colloque
218 international du CNRS 219, 203-210.

Stevens, C.H., Jones, D.H., Todd, R.G., 1960. Ultrasonic vibrators as a cleaning agent for fossils.
220 *Journal of Paleontology* 34, 727-730.

Urey, H.C., 1947. The thermodynamic properties of isotopic substances. *Chemical Society
222 (London) Journal*, 562-581.

Waelbroeck, C., Mulitza, S., Spero, H., Dokken, T., Kiefer, T., Cortijo, E., 2005. A global
 224 compilation of late Holocene planktonic foraminiferal $\delta^{18}\text{O}$: relationship between surface water
 temperature and $\delta^{18}\text{O}$. *Quaternary Science Reviews* 24, 853-868.

226 Walker, D.A., Linton, A.E., Schafer, C.T., 1974. Sudan Black B: A superior stain to Rose Bengal
 for distinguishing living from nonliving foraminifera. *Journal of Foraminiferal Research* 4, 205-
 228 215.

Walton, W.R., 1952. Techniques for recognition of living foraminifera: Contributions
 230 from the Cushman Foundation for Foraminiferal Research 3, 56-60.

Wefer, G., Berger, W.H., 1991. Isotope paleontology: Growth and composition of extant
 232 calcareous species. *Marine geology* 100 (1-4), 207-248.

Wierzbowski, H., 2007. Effects of pre-treatments and organic matter on oxygen and carbon isotope
 234 analyses of skeletal and inorganic calcium carbonate. *International Journal of Mass Spectrometry*
 268, 16-29.

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Figure 1. $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ values of treated and untreated foraminifera samples. The
 238 vertical bars indicates standard error of mean. Horizontal dotted lines represents the
 mean isotopic values of untreated samples. US: ultrasonic cleaning; BR: Bengal Rose
 240 stain; SB: Sudan Black B stain. Asterisks indicate significant differences respect to
 untreated samples (T-test, * $P < 0.05$).

Table 1. Changes in δ values of pre-treated foraminifera samples relative to the untreated samples ($\Delta \delta^{13}\text{C}$). N is the number of replicates; S.E.M. is the standard error of the mean; n is the number of individuals per replicate. Significant deviations are indicated by an asterisk (T-test, $P < 0.05$).

Treatment	$\delta^{13}\text{C}_{\text{PDB}}\text{‰}$			$\delta^{18}\text{O}_{\text{PDB}}\text{‰}$			n
	N	$\Delta \delta^{13}\text{C}$ (‰)	S.E.M.	N	$\Delta \delta^{18}\text{O}$ (‰)	S.E.M.	
Bengal Rose	4	+0.02	0.05	4	+0.12	0.01	4
Sudan Black B	3	+0.24*	0.03	3	-0.07	0.03	4
Formalin	3	+0.09	0.07	3	+0.07	0.02	4
Ethanol	4	+0.01	0.04	4	-0.14	0.04	4
Bengal Rose & Formalin	3	+0.06	0.05	3	+0.11	0.04	4
Bengal Rose& Ethanol	4	+0.09	0.04	4	-0.08	0.03	4
Plasma ashing	3	-0.04	0.03	3	-0.04	0.02	4
Sodium hypochlorite	3	+0.09	0.05	3	+0.02	0.05	4
Hydrogen peroxide	3	-0.08	0.12	3	-0.18*	0.13	4
Ultrasonic cleaning	4	+0.03	0.05	4	-0.18*	0.03	4
Sodium hypochlorite & Ultrasonic	4	+0.05	0.04	4	+0.05	0.03	4

Hydrogen peroxide & Ultrasonic	4	+0.02	0.03	4	-0.04	0.01	4
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