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RESEARCH ARTICLE

Effect of removal of organic material on stable isotope ratios in skeletal carbonate from taxonomic groups with complex mineralogies

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Rationale: Stable oxygen and carbon isotope ratios are one of the most accurate ways of determining environmental changes in the past, which are used to predict future environmental change. Biogenic carbonates from marine organisms are the most common source of samples for stable isotope analysis. Before they are analyzed by mass spectrometry, any organic material is traditionally removed by one of three common pretreatment methods: roasting, bleaching, or with hydrogen peroxide at various strengths and durations.

Methods: This study compares $\delta^{18}O$ and $\delta^{13}C$ values in a control with no pretreatment with those from five different pretreatment methods using conventional acid digestion mass spectrometry. The objectives are to: assess the impact of the most common pretreatment methods on $\delta^{18}O$ and $\delta^{13}C$ values from (1) taxonomically underrepresented groups in previous studies, and (2) those that precipitate a wide range of biomineralogies, in the debate of whether to pretreat or not to pretreat. We analyzed the following biomineralogically complex temperate marine organisms from southern New Zealand: four species of bryozoans, four species of molluscs, two species of arthropods, and one species each of annelid, red alga, brachiopod, and echinoderm (test plates and spines treated separately). These species precipitate aragonite, High-, Intermediate-, and/or Low-Mg calcite (LMC) in their skeletons. We used linear mixed statistical models to compare the effects of the pretreatments and mineralogical composition on their $\delta^{18}O$ and $\delta^{13}C$ values.

Results: Roasting was the most effective pretreatment for the removal of organic matter and light H_2O_2 the least, but the former had corresponding impacts on isotope ratios. $\delta^{18}O$ values were not directly affected by wt% $MgCO_3$, but they were significantly affected by the interaction of roasting and wt% $MgCO_3$. This same negative effect of roasting on species with higher wt% $MgCO_3$ occurred in $\delta^{13}C$ values, but it was much more pronounced in $\delta^{18}O$ values. Both H_2O_2 pretreatments significantly and negatively affected $\delta^{18}O$ values at higher wt% $MgCO_3$. Neither bleaching pretreatment significantly affected $\delta^{18}O$ values. $\delta^{13}C$ values were most negatively affected in skeletons with high wt% $MgCO_3$. There was also a strong negative roasting effect and more so at higher wt% $MgCO_3$. Bleaching and H_2O_2 did not significantly affect $\delta^{13}C$ values.

Conclusions: Based on these results, and when using skeletal carbonate of complex mineralogies, we recommend considering the abandonment of pretreatment of biogenic carbonate for stable isotope analysis due to confounded results from previous studies, difficulties with preparation, and/or the absence of significant effects of organic material on isotope ratios. If pretreatment is necessary, avoid roasting especially at higher temperatures and durations, use minimal bleaching, and in general avoid using High-Mg calcite species in O and C stable isotope studies. If bleaching is used, clearly indicate the concentration and duration of exposure.

1 | INTRODUCTION

The discovery of a correlation between stable isotopic variations preserved in ancient carbonates and a variety of environmental parameters has enabled researchers to reconstruct environmental change over Earth history.[1] A clear understanding of past climate allows more accurate predictions of future environmental change.[2] Perhaps the most important paleothermometer, McCrea's (1950) oxygen (O) isotope equation, allows for the determination of temperature based on the O isotope composition of ancient carbonate minerals.[3] Suitable carbonate minerals that grow by accretion, and thus preserve temporal change, range from speleothem-derived abiogenic carbonates that record climate change to fish-derived biogenic carbonates that record migratory patterns.[4–7] Likewise, information from stable carbon (C) isotope ratios in natural carbonates has made fundamental contributions to the study of Earth history and processes from temporal changes in productivity to the planet's C budget.[8–10] Finally, O and C stable isotope analysis has been applied to diagenetic carbonate cements which occlude porosity and permeability in petroleum reservoirs.[11,12]

Many analytical geochemical techniques require pretreatment of samples before analysis.[13,14] In the older literature, the term "cleaning technique" is often used instead of the more common use of "pretreatment method" today. Pretreatment of biogenic $CaCO_3$, in particular, was traditionally performed to remove the organic matter because much biogenic $CaCO_3$ consists of both mineral and organic fractions.[15] The organic matrix is finely disseminated both within and between the $CaCO_3$ crystals and cannot be easily removed by physical means.[16–19]

Pretreatment of biogenic carbonates for O and C isotope analysis using conventional acid digestion followed by mass spectrometry is aimed at preventing the reaction between any organic matter and H_3PO_4 (phosphoric acid) used to liberate CO_2 from the skeletal carbonate.[20,21] Organics also tend to inhibit complete dissolution of the carbonate material during isotopic analysis.[22] Epstein et al[23] noted that, during the H_3PO_4 acid extraction of CO_2 from biogenic carbonate, the O and C isotope composition of the liberated CO_2 gas was contaminated by the presence of organic matter. When measuring the O and C isotope compositions of skeletal carbonate, the goal is to measure the signature in the mineral fraction, which must be separated from the coexisting organic material. This separation is necessary because stable isotopes are differently fractionated in the organic and inorganic fractions of skeletal tissues.[24] Epstein et al[23] stated that the organic matter should be removed to allow measurement of the pristine isotope composition of the skeletal carbonate. They therefore proposed a pretreatment process to remove the organic matter by roasting the sample, and the rest is history.

Although Epstein et al[25] were the first to introduce pretreatment of biogenic carbonates by roasting, their later experiments on isotope exchange between gaseous CO_2 and biogenic carbonate provided no clear evidence for a source of observed changes in $\delta^{18}O$ values.[23,26] Roasting removes volatile components in the organic matter which may interact with the generated CO_2, so decreases in $\delta^{18}O$ and $\delta^{13}C$ values measured in biogenic carbonates after pretreatment have been attributed to isotope exchange processes.[27–32] Some studies used a known abiotic carbonate standard, added some known organic material to it, and then analyzed it to see what the interaction is between the phosphoric acid and the organics.[33,34] But experiments involving abiotic pure $CaCO_3$ mixed with organic material show no effect on the original isotope ratios.[26,32,35,36] Measured decreases in the δ values of skeletal carbonates following pretreatment may be linked to removal of organic contaminants, alteration of mineral phases, or isotopic exchange with organics or other substances occurring during pretreatments.[26] This is why abiotic pure carbonates are not normally used as real controls in the study of the effects of pretreatment methods on biogenic carbonate.

There have been numerous studies examining the effects of various pretreatments to remove organic material on O and C stable isotope ratios in biogenic carbonate. This study is restricted to common conventional acid digestion mass spectrometry as opposed to alternative approaches such as laser ablation.[37] The most commonly used pretreatment methods are thermal roasting, bleaching with sodium hypochlorite, and oxidizing with hydrogen peroxide.[38] Only 34 of these studies have quantified the effects of pretreatment on O and C stable isotope ratios with a variety of different organisms, mineralogies, concentrations/temperatures, and exposure durations (Table 1). These studies include some that utilized fossil biogenic carbonate.[33,40,41,49,60]

The most common pretreatment method is thermal roasting at a temperature which ranges from 80 to 700°C, typically for ~1 h (Table 1). The original method of organic removal was roasting in helium.[25] The second-generation method was roasting *in vacuo*.[61]

TABLE 1 Results from previously published studies on the effects on $\delta^{18}O$ and $\delta^{13}C$ values of various pretreatments to remove organic material from biogenic carbonates using conventional acid digestion mass spectrometry. The delta values are the mean deviation from the control. If the mean was not reported, it was calculated from the published values. Bold indicates that the effect was considered significant, italics indicate insignificant, neither indicated nor stated by the original author(s). Results sorted by year of publication

			NaOCl (sodium hypochlorite, bleach)				H_2O_2 (hydrogen peroxide)				Vacuum or helium roasting			
Study	Source	Mineralogy	Concentration (%)	Duration (h)	$\Delta\delta^{18}O$ (‰)	$\Delta\delta^{13}C$ (‰)	Concentration (%)	Duration (h)	$\Delta\delta^{18}O$ (‰)	$\Delta\delta^{13}C$ (‰)	Temperature (°C)	Duration (h)	$\Delta\delta^{18}O$ (‰)	$\Delta\delta^{13}C$ (‰)
Epstein et al[25]	Bivalves	Calcite and aragonite									400	1	**−1.4**	
Epstein et al[25]	Brachiopods	Calcite									400	1	**−1.1**	
Epstein et al[25]	Gastropods	Calcite and aragonite									400	1	**−1.5**	
Epstein et al[23]	Gastropods	Aragonite									470	0.5	**0.8**	
Lowenstam and Epstein[39]	Algae	Aragonite	5.3		*0.0*	*0.1*								
Emiliani[40]	Bivalves	Calcite and aragonite			0.0						475		−0.2	
Emiliani[40]	Belemnites	Calcite									475		−0.4	
Emiliani[40]	Foraminifera	Calcite			0.0						475		0.0	
Weber and Raup[41]	Echinoids	Calcite	5.25	48	*−0.03*	*0.02*								
Mook[42]	Bivalves	Calcite and aragonite									470	0.5	*−0.56*	*−0.20*
Forester et al[27]	Bryozoans	Intermediate- to high-Mg calcite	1	0.5	*0.11*	*0.01*					425	0.33	**−2.03**	**−0.35**
Forester et al[27]	Bryozoans	Intermediate- to high-Mg calcite	1	1	*0.10*	*0.23*								
Savin and Douglas[43]	Foraminifera	Calcite	10	24	−0.29	−0.20								
Land et al[28]	Corals	Aragonite	5	8	−0.29	−0.20					470	0.5	−0.87	−0.19
Durazzi[44]	Ostracods	Calcite	5	24	*−0.04*	*0.01*								
Durazzi[44]	Ostracods	Calcite	5	120	*−0.05*	**−0.27**								
Erez and Honjo[29]	Foraminifera	Calcite									400	0.5	−0.22	−0.09
Erez and Honjo[29]	Foraminifera	Calcite									400	1	−0.20	−0.12
Grossman et al[45]	Gastropods	Aragonite	5	16	*0.07*	*−0.01*					470	1	**0.69**	*0.02*
D'Eugenio and Leone[46]	Bivalves & gastropods	Calcite with minor aragonite	5	12	**−0.58**	**−0.76**	35	24	−0.05	−0.12	390		−0.16	−0.03
D'Eugenio and Leone[46]	Bivalves & gastropods	Aragonite with no or minor calcite	5	12	−0.25	−0.25	35	24	0.25	−0.11	390		−0.20	−0.19

(Continues)

TABLE 1 (Continued)

			NaOCl (sodium hypochlorite, bleach)				H_2O_2 (hydrogen peroxide)				Vacuum or helium roasting			
Study	Source	Mineralogy	Concentration (%)	Duration (h)	$\Delta\delta^{18}O$ (‰)	$\Delta\delta^{13}C$ (‰)	Concentration (%)	Duration (h)	$\Delta\delta^{18}O$ (‰)	$\Delta\delta^{13}C$ (‰)	Temperature (°C)	Duration (h)	$\Delta\delta^{18}O$ (‰)	$\Delta\delta^{13}C$ (‰)
McConnaughey[35]	Corals	Aragonite							*0.08*	*0.02*	275		*0.08*	*0.05*
Sarkar et al[36]	Foraminifera	Calcite									400	1.5	*−0.09*	*−0.03*
Bowen[47]	Serpulids	Calcite									470	0.5	**−0.90**	
Bowen[47]	Gastropods	Aragonite									470	0.5	**−0.60**	
Boiseau and Juillet-Leclerc[30]	Corals	Aragonite					30	12	−0.17	−0.35	350	2	**−0.57**	*−0.20*
Hickson et al[48]	Bivalves	Calcite									350	1	*−0.3*	*−0.1*
Leone et al[49]	Gastropods	Aragonite									400	1	*−0.11*	*0.14*
Guiguer et al[50]	Fish otoliths	Aragonite									275	1	*−0.18*	*−0.07*
Bice and Norris[51]	Foraminifera	Calcite	5.25	3	*0.1*	*−0.1*	3	17	*0.1*	*−0.1*				
Grottoli et al[52]	Corals	Aragonite	5.25	24	**−0.09**	**−0.2**	30	24	*−0.04*	**−0.05**				
Keatings et al[53]	Ostracods	Calcite	5	4	−0.6	−0.5	5	0.25	*0.0*	−0.5	380	1	−0.4	−0.6
Li et al[54]	Ostracods	Calcite							*0.0*	*−0.1*				
Wierzbowski[26]	Molluscs	Aragonite									200	0.75	**−0.20**	*0.00*
Wierzbowski[26]	Molluscs	Aragonite and calcite	5	18	**−0.20**	**−0.26**	30	12	*0.10*	*0.04*	345	0.75	*−0.17*	*−0.03*
Wierzbowski[26]	Molluscs	Aragonite and calcite									450	0.75	*−0.05*	**−0.05**
Denniston et al[55]	Corals	Aragonite									200	1	*0.05*	**0.20**
Serrano et al[56]	Foraminifera	Calcite	10	0.17	*0.02*	*0.09*	30	0.17	**−0.18**	*−0.08*				
Nagtegaal et al[57]	Corals	Aragonite	6.25	24	*0.01*	*0.04*								
Feldmeijer et al[20] supplemental data table A6	Foraminifera	Calcite					10	1	*−0.05*	*0.03*				
Milano et al[58]	Gastropods	Aragonite									300	0.67	−0.5	
Milano et al[58]	Gastropods	Aragonite									500	0.67	−1.1	
Milano et al[58]	Gastropods	Aragonite									700	0.67	−1.3	
Meyer et al[59]	Ostracods	Calcite					10	1.4	*0.15*	*0.08*				
Schöne et al[33]	Bivalve	Aragonite	12		*0.09*	*−0.19*	30		**0.10**	*−0.05*				
Roberts et al[60]	Ostracods	Calcite	5	4	**−0.12**	**−0.07**	15	0.25	*−0.005*	*−0.003*	80	3	*−0.03*	*0.02*
Roberts et al[60]	Ostracods	Calcite	5	24	**−0.17**	**−0.13**	30	0.5	*−0.02*	*0.02*	250	1	**−0.09**	*−0.04*
Zhang et al[34]	Corals	Aragonite					10	18	**0.63**	**0.55**				
Zhang et al[34]	Corals	Aragonite					10	48	**0.59**	**0.57**				
Zhang et al[34]	Gastropods	Aragonite					10	18	**0.50**	**0.45**				

(Continues)

TABLE 1 (Continued)

Study	Source	Mineralogy	NaOCl (sodium hypochlorite, bleach)				H_2O_2 (hydrogen peroxide)				Vacuum or helium roasting			
			Concentration (%)	Duration (h)	$\Delta\delta^{18}O$ (‰)	$\Delta\delta^{13}C$ (‰)	Concentration (%)	Duration (h)	$\Delta\delta^{18}O$ (‰)	$\Delta\delta^{13}C$ (‰)	Temperature (°C)	Duration (h)	$\Delta\delta^{18}O$ (‰)	$\Delta\delta^{13}C$ (‰)
Zhang et al[34]	Gastropods	Aragonite					10	48	**0.46**	**0.57**				
Zhang et al[34]	Bivalves	Aragonite					10	18	**0.23**	*0.13*				
Zhang et al[34]	Bivalves	Aragonite					10	48	**0.33**	*0.18*				
		Minimum	1.0	0.2	−0.60	−0.76	3	0.2	−0.18	−0.50	80	0.3	−2.03	−0.60
		Median	5.0	14.0	−0.04	−0.12	10	17.5	0.10	0.02	400	1.0	−0.20	−0.05
		Mean	5.6	20.4	−0.10	−0.13	19	17.5	0.14	0.06	391	0.9	−0.42	−0.09
		Maximum	12.0	120.0	0.11	0.23	35	48.0	0.63	0.57	700	3.0	0.80	0.20
		Standard deviation	2.5	27.0	0.20	0.22	11	16.2	0.23	0.27	110	0.5	0.58	0.17

Roasting, especially at higher temperatures, typically leads to more negative isotope ratios, especially for oxygen (Table 1). This has been attributed to isotope exchange with internal water in the minerals or more commonly to conversion of unstable forms of $CaCO_3$ (i.e., aragonite, High-Mg Calcite (HMC), and Intermediate-Mg Calcite (IMC)) into the more stable Low-Mg Calcite (LMC).[26,30]

The second most commonly used pretreatment method is bleaching with sodium hypochlorite, usually 5% NaOCl for ~20 h (Table 1). This method was first applied in isotope studies by Lowenstam and Epstein.[39] Bleaching typically leads to only slightly more negative isotope ratios (Table 1). This has been attributed to isotope exchange with the reagent and/or precipitation of $Ca(OH)_2$.[26,52,53]

The most recently applied pretreatment method is immersion in hydrogen peroxide,[35] and it usually involves soaking in 10% H_2O_2 for ~10 h (Table 1). H_2O_2 treatment typically leads to insignificant changes in isotope ratios (Table 1). This has been attributed to isotope exchange with the reagent and/or partial dissolution of carbonate.[26,30,34,52,53,56] The effect of the pretreatment is proportional to the treatment duration. The mean H_2O_2 treatment duration used by Zhang et al[34] was three times longer than that of all the other studies combined (i.e., 33 h vs 10 h). This could explain the significantly positive effect on both their $\delta^{18}O$ and $\delta^{13}C$ values (i.e., mean: +0.46‰ and +0.41‰, respectively) compared with all the other studies combined which are consistently minimal and insignificant (i.e., mean: +0.02‰ and −0.08‰, respectively).

Only two studies have measured the effect of low-temperature plasma ashing to oxidize the organic material in biogenic carbonate.[29,53] However, as that technology was unavailable for this study, the plasma ashing pretreatment method was not included in this investigation.

One obvious pattern in the previously published studies is the organisms used (Table 1); over half of the studies examine monomineralic tropical aragonite corals and calcite foraminifera and ostracods. Temperate carbonate-producing macrofaunal organisms that dominate in cool-to-cold waters (i.e., mollusks, bryozoans, echinoids, barnacles, and serpulid worms)[62] are noticeably underrepresented. Another general pattern in these studies (Table 1) is the lack (~20%) of taxa with complex and diverse skeletal mineralogy (e.g., bryozoans), as most studies used monomineralic species. It is time to apply rigorous analysis of the effects of the various pretreatments on other taxonomic groups such as temperate invertebrates with diverse mineralogies, especially bryozoans.

Bryozoans precipitate pure calcite, pure aragonite, various degrees of HMC, IMC, and LMC, as well as a continuum of aragonite-calcite combinations.[63,64] Due to the extraordinary biomineralogical diversity in bryozoans, it is important that we understand the effects of the various pretreatments on their O and C stable isotope ratios. This study will improve the analysis of O and C stable isotope ratios from these underrepresented, mineralogically complex, cool-water carbonate producers, and compare their response with those of some temperate monomineralic species.

Therefore, the objectives of this study are to: assess the impact of the most common pretreatment methods on $\delta^{18}O$ and $\delta^{13}C$ values

from (1) taxonomically underrepresented groups, and (2) those that precipitate a diverse range of biominerals in the debate on whether to pretreat or not to pretreat.

2 | EXPERIMENTAL

2.1 | Previous pretreatment methods

The most common experimental approach to evaluating the effects of pretreatments on isotope ratios derived from biogenic carbonate involves comparing splits of pretreated and untreated samples from a preferred group of organisms (Table 1). The benefit of comparing splits of pretreated and untreated samples is that it avoids other sources of variation in isotope ratios from species growing in different water chemistry and temperature and those with vital effects.[35,65–68] However, this approach cannot fully resolve the problem because, if a change were observed in the isotope ratios, it could be due to the pretreatment's removal of organic material, alteration of the isotope ratios of the carbonate material itself by the pretreatment, or some combination of these. On the other hand, if no change were observed, offsetting chemical changes may have occurred from the removal of elements with the organic material and the gain of those elements from the pretreatment, or the method may have been ineffective at removing organic material. Because these problems may preclude the effective use of biogenic samples for testing the hypothesis that pretreatments intended to remove organic material also alter carbonate chemical compositions, splits of pure abiogenic carbonate standards are sometimes used.[26,36,69,70] This approach removes one variable from the experiments (i.e., the presence of organic matter), but it is less commonly used as it fails to address the potential impact of any remaining organic matter. In addition, biogenic carbonate (at least aragonite) is more thermally sensitive to inversion to calcite than abiogenic aragonite,[71] so using abiogenic carbonate may not be a valid substitute.

This study takes a slightly different approach from the more common experimental pretreated vs untreated approach and looks for general patterns (e.g., increasing or decreasing $\delta^{18}O$ or $\delta^{13}C$ values or their ranges of variation) among a taxonomically diverse group of biogenic carbonates with varying mineralogies. The null hypothesis to be tested is that roasting, bleaching, and hydrogen peroxide pretreatments which remove organic material do not affect the O and C stable isotope ratios in carbonate organisms at a scale detectable by mass spectrometry. The materials and methods used in this study mirror those from a parallel study by Smith et al[72] on the effects of pretreatment on mineralogy as determined by X-ray diffraction (XRD).

2.2 | Samples

Biogenic skeletal carbonate was collected from a range of temperate marine environments around southern New Zealand, particularly the Otago Shelf and Stewart Island (Table 2). Instead of just choosing one carbonate biomineralizing species, multiple species from diverse groups were selected, since it has been shown that the effects of pretreatment can vary among taxa.[73] Dominant temperate, carbonate-sediment-producing taxa were selected for this study.[62,74,75] Fourteen species from New Zealand were collected, including bryozoans (4 species), bivalve molluscs (3 species), barnacles (2 species), coralline red alga, serpulid worm, gastropod mollusc, brachiopod, and an echinoid (1 species each) (Table 2). Due to the different mineralogy of the echinoid's test plates and spines (Table 2),[41,76] these skeletal components were sampled separately for a total of 15 "species".

These are the same species as used by Smith et al,[72] although we excluded two of their species and added an additional one. We did not use the bivalves *Atrina zelandica* and *Cardita distorta*, but we added the gastropod *Haliotis iris*. The two bivalves were excluded from this study because Smith et al[72] found them mineralogically inconsistent and thus excluded them from statistical analysis. We added the haliotid gastropod to include a bimineralic species from this diverse class of molluscs and because this genus was used in many of the pioneering pretreatment studies.[23,25,77] The species chosen represent a wide range of biominerals in their skeletons, including: calcite (<1% $MgCO_3$), LMC (1–4 wt% $MgCO_3$), IMC (4–8 wt% $MgCO_3$), HMC (>8 wt% $MgCO_3$), aragonite, and mixtures of aragonite and calcite (Table 2).

2.3 | Sample collection

Un-encrusted and un-bored specimens were collected (by hand or by dredge), either living or freshly dead, and not exposed to chemicals or preservatives of any kind after collection. Where possible, the organism was scraped out without damaging the shells, which were then rinsed in distilled water at room temperature (i.e., 15–17°C). For each species, three clean individual specimens (or colonies, in the case of bryozoans) were selected. Each specimen was cut (using a cold hack-saw) or snapped into six pieces weighing ~5 g each. We avoided using a power saw to reduce frictional heating which may decrease or increase O and C isotope ratios but not always significantly.[78–83] Each piece was given a unique identifier and assigned to one of six pretreatments. Each species was thus represented by three replicates in each pretreatment. All specimens were kept at room temperature in a desiccator until ready for pretreatment to minimize isotopic exchange with atmospheric water vapor. Immediately prior to pretreatment, specimens (except the coralline alga and the sea urchin spines) were weighed and photographed.

2.4 | Pretreatment methods to remove organic matter

From the commonly used pretreatment methods for removal of organic matter from carbonates that are frequently used and reported in the literature (Table 1), six pretreatments were selected. (1) Control: The controls are not "controls" in the classical sense as their isotope

TABLE 2 Mineralogy and source of species collected from New Zealand for the biogenic carbonate used in this study, arranged in order of increasing mineralogical stability

Species	Taxonomy (phylum: Class: Order: Family)	Mean weight % calcite; the remaining % is aragonite	Mean weight % $MgCO_3$	Mineralogical grouping	Sample location	Latitude (°S)	Longitude (°E)	Water depth (m)
Barbatia novaezealandiae (E.A. Smith, 1915)	Mollusca: Bivalvia: Arcida: Arcidae	0	0.0	Aragonite	Bench Island, Paterson inlet, Stewart Island	46.9	168.24	47
Mytilus galloprovincialis Lamarck, 1819	Mollusca: Bivalvia: Mytilida: Mytilidae	72	1.9	Low-Mg calcite + up to 34% aragonite	Port Pegasus, Stewart Island	47.24	167.62	Intertidal
Galeolaria hystrix Mörch, 1863	Annelida: Polychaeta: Sabellida: Serpulidae	97	9.8	High-Mg calcite + up to 4% aragonite	Paterson inlet, Stewart Island	46.96	168.13	8
Haliotis iris Gmelin, 1791	Mollusca: Gastropoda: Vetigastropoda: Haliotidae	Relative mix of aragonite and calcite varies widely with the shell layer sampled		Low-Mg calcite + aragonite	Lords river, Stewart Island	47.12	168.15	3
Arthrocardia corymbosa (Lamarck) Decaisne, 1842	Rhodophyta: Florideophyceae: Corallinales: Corallinaceae	100	12.1	High-Mg calcite	Paterson inlet, Stewart Island	46.87	168.15	Intertidal
Evechinus chloroticus (Valenciennes, 1846) (test plates)	Echinodermata: Echinoidea: Camarodonta: Echinometridae	100	8.5	High-Mg calcite	Lords river, Stewart Island	47.12	168.15	3
Hippomenella vellicata (Hutton, 1873)	Bryozoa: Gymnolaemata: Cheilostomata: Schizoporellidae	100	7.8	Intermediate-Mg calcite	Otago shelf	45.67	170.96	70
Celleporaria emancipata Gordon, 1989	Bryozoa: Gymnolaemata: Cheilostomata: Lepraliellidae	100	7.4	Intermediate-Mg calcite	NW of Snares Islands	47.52	166.71	168
Evechinus chloroticus (Valenciennes, 1846) (spines)	Echinodermata: Echinoidea: Camarodonta: Echinometridae	100	4.8	Intermediate-Mg calcite	Lords river, Stewart Island	47.12	168.15	3
Hornera foliacea (MacGillivray, 1869)	Bryozoa: Stenolaemata: Cyclostomata: Horneridae	100	1.5	Low-Mg calcite	Otago shelf	45.67	170.96	70
Hornera robusta MacGillivray, 1883	Bryozoa: Stenolaemata: Cyclostomata: Horneridae	100	1.3	Low-Mg calcite	Otago shelf	45.67	170.96	70
Austromegabalanus psittacus (Molina, 1782)	Arthropoda: Maxillopoda: Sessilia: Balanidae	100	0.9	Calcite	Sailors rest, Paterson inlet, Stewart Island	46.97	168.14	15
Pecten novaezelandiae Reeve, 1852	Mollusca: Bivalvia: Pectenida: Pectenidae	100	0.6	Calcite	Port Pegasus, Stewart Island	47.21	167.62	17
Elminius modestus Darwin, 1854	Arthropoda: Maxillopoda: Sessilia: Austrobalanidae	100	0.5	Calcite	Port Pegasus, Stewart Island	47.24	167.62	Intertidal
Calloria inconspicua (Sowerby, 1846)	Brachiopoda: Rhynchonellata: Terebratulida: Terebratellidae	100	0.3	Calcite	Bench Island, Paterson inlet, Stewart Island	46.87	168.21	43

ratios are unknown. We refer to them as controls here as they were untreated compared with the other five pretreatments. This use of controls follows the terminology of previous studies (Table 1) and the parallel XRD study.[72] (2) Roasting: Roast in a muffle furnace at 550°C for 1.5 h. We chose a roasting temperature (550°C) which is well above the mean temperature of previous studies (391°C, range: 200–700°C in Table 1). We chose such a high temperature as we wanted to clearly document the isotopic effect of the aragonite-to-calcite transformation in our aragonite species (Table 2). The fundamental experiments of Davis and Adams show that the conversion of aragonite into calcite occurs continuously with increasing time and temperature of roasting until a temperature-specific equilibrium is reached.[84] For example, at 380°C, <50% of aragonite has transformed to calcite after 10 h.[84] In gastropods with bilaminar aragonite skeletons, the carbonate alters to calcite at 300–500°C.[58] Our 550°C roasting for 1.5 h ensured that all the aragonite and HMC had converted into LMC. Roasting at 200°C may certainly be appropriate for monomineralic calcitic tropical taxa (Table 1), but we needed a higher temperature for these mineralogically complex taxa. (3) Light Bleach: Immerse in 30% bleach (i.e., 7 g/L NaOCl, sodium hypochlorite) for 3 h. (4) Hard Bleach: Immerse in 100% bleach (i.e., 21 g/L NaOCl) for 3 days, stirring several times. (5) Light H_2O_2: Immerse in hydrogen peroxide (5% H_2O_2) for 3 h. (6) Hard H_2O_2: Immerse in hydrogen peroxide (30% H_2O_2) for 3 days, stirring several times.

After pretreatment, all specimens were rinsed three times in distilled water, air dried at room temperature at ~15°C, then kept in a desiccator until being weighed. Each specimen was weighed to calculate the mean weight loss resulting from pretreatment. After weighing, each specimen was subsampled and a 2 g piece was kept for archive. To avoid the influence of varying grain size (a.k.a., crystallinity) on stable isotope analysis,[85] a 3 g piece was hand-ground in an agate mortar to a crystallite powder for O and C stable isotope analysis. The powders were homogenized to minimize any potential effects of specimen-related variation in isotope ratios.[67,86] Thus, for this study, there was an initial total of 270 samples consisting of 15 species, three replicates, and six pretreatments. For quality control of isotope analyses, roughly every eighth powder sample was replicated, for a total of 300 samples.

The following sample preparation was performed at Chemical Solutions Ltd (Harrisburg, PA, USA). On average 244 μg (range: 180–420 μg; standard deviation (SD): 52 μg) of carbonate powder was measured out on a XS225 Dual range digital balance (Mettler Toledo, Columbus, OH, USA) to the nearest 10 μg. Each sample was placed in a 12.0-mL round-bottomed borosilicate vial (Exetainer®, 101 mm high × 15.5 mm diameter; Labco, Lampeter, UK) with an impermeable pierceable chlorobutyl septum lid.

2.5 | Stable isotope ratio mass spectrometry

The measurement of $\delta^{18}O$ and $\delta^{13}C$ values was performed at the Iso-trace Research laboratory in the Department of Chemistry, University of Otago, New Zealand. Each vial was ultrasonicated for 15 min to settle the powder into the acid well at the bottom of the vial. Standard carbonates were weighed to 200 ± 20 μg to ensure that all tests provided similar signal intensity. Labels were individually checked and lids retightened while the vials were being placed into the autosampler tray. Air was flushed from the closed vials with helium and 4–5 drops of 105% anhydrous phosphoric acid injected through the septum. Acid and carbonate were reacted for 18 h at 25.00 ± 0.01°C to produce CO_2 gas. Both the flushing and the sampling of vials were performed by a Thermo GasBench automated preparation system (Thermo Fisher Scientific, Bremen, Gertmany). Ten replicate 100-μL aliquots of the CO_2 + He headspace gas were injected into a Thermo Advantage isotope ratio mass spectrometer bracketed by pulses of CO_2 monitoring gas. Raw delta values were calculated using four monitoring gas pulses and the 10 results filtered by removing any result more than one SD from the mean. The means of the filtered raw deltas were normalized to the international delta scale (Vienna Pee Dee Belemnite, VPDB) using three carbonate standard materials measured at the beginning of each of the five batches of samples. The standards comprised two international standards (NBS-18 and NBS-19) and one laboratory standard (IRU-Marble) which has been repeatedly standardized against international standards. Every eighth sample was replicated for quality control testing against these standards. Drift control materials were analyzed at every 12th position and were used to calculate instrumental drift per unit time by linear regression. Drift correction was applied if this regression showed a correlation coefficient greater than 0.7. The control materials BORBA and ATLANTIS3 are marine carbonates that were determined by an interlaboratory comparison (University College London, London, UK) in 2008. Mass dependence was assessed with three samples of IRU-Marble weighing between 100 and 450 μg. No corrections were needed for mass dependence during this work. The precision of the $\delta^{18}O$ and $\delta^{13}C$ values was ±0.07‰VPDB and ±0.04‰VPDB, respectively. Isotope ratios are reported in per mil notation (‰) relative to the VPDB scale using the standard δ notation where: $\delta_{sample} = (R_{sample}/R_{standard} - 1)$. R is the $^{18}O/^{16}O$ ratio or the $^{13}C/^{12}C$ ratio.

2.6 | Statistical analysis methodology

We used linear mixed models to examine the effects of the pretreatments and mineralogical composition on $\delta^{18}O$ and $\delta^{13}C$ values. Our fixed effects were pretreatment and mineralogy. To accommodate the repeated-measures design of the study, a random intercept and slope effect were fitted for each of the specimens included in the analysis. Models were analyzed using the R-library package Linear and Non-Linear Mixed Effects Models.[87] Contrast plots and confidence intervals were constructed using the R-library package Visualization of Regression Functions.[88]

To examine the effect of mineralogy on $\delta^{18}O$ and $\delta^{13}C$ values, we used the wt% $MgCO_3$ in calcite data from the parallel study by Smith et al.[72] This required excluding two of the species from the model.

One is the all-aragonite bivalve *Barbatia novaezealandiae* which by default can not contain any wt% $MgCO_3$ in calcite as it is 100% aragonite. The other is the gastropod *Haliotis iris* whose relative mix of aragonite and calcite varies so widely with the shell layer sampled that it did not yield robust wt% $MgCO_3$ results.[72]

3 | RESULTS

Two samples had chipped vial lids and the CO_2 gas was lost due to vial leakage, so a total of 298 samples were successfully analyzed for $\delta^{18}O$ and $\delta^{13}C$. Mean differences in $\delta^{18}O$ and $\delta^{13}C$ values from the controls for each species after each pretreatment are reported in Table 3. The complete dataset is available in Table S1 (supporting information).

All pretreatments resulted in weight loss as measured in the difference in percentage from the control (Figure 1, Table 4). All but two of the controls had <1% wt% loss (Figure 1, Table 4), indicating the amount of background noise from mechanical loss. All treatments resulted in statistically significant (t-test, $p < 0.05$) weight loss except the Light H_2O_2 pretreatment (t-test, $p = 0.29$). In all species, Roasting was the most effective pretreatment for removal (mean wt% loss: 5.5), followed by Hard H_2O_2 (mean wt% loss: 3.0) and then Hard Bleach (mean wt% loss: 2.5) (Table 4). In both cases (i.e., Bleach and H_2O_2), the Hard pretreatment removed more material than the Light pretreatment (Figure 1, Table 4).

As indicated in Figure 2, the $\delta^{18}O$ values are less variable overall than the $\delta^{13}C$ values. The $\delta^{18}O$ values ranged from −4.13 to 2.12‰ VPDB (mean: 0.50‰VPDB, SD: 0.97‰VPDB), and the $\delta^{13}C$ values from −7.83 to 2.36‰VPDB (mean: 0.01‰VPDB, SD: 1.93‰VPDB). Without accounting for the effects of pretreatment and mineralogy, four species stand out (Figure 2). The coralline alga *Arthrocardia corymbosa* has the most negative and variable $\delta^{18}O$ and $\delta^{13}C$ values (Figure 2A; mean: −1.77‰VPDB and −5.04‰VPDB, respectively; SD: 1.23‰VPDB and 1.16‰VPDB, respectively). It is also the species with the highest wt% $MgCO_3$ (Table 2). The test plates of the sea urchin *Evechinus chloroticus* have very negative $\delta^{13}C$ values (Figure 2B; mean: −3.84‰VPDB). The serpulid *Galeolaria hystrix* has quite negative $\delta^{13}C$ values (Figure 2C; mean: −0.95‰VPDB), and the clam *Barbatia novaezealandiae* has slightly more positive $\delta^{13}C$ values (Figure 2D; mean: 2.11‰VPDB).

The statistical model predicted the stable isotope ratios from the direct effects of the various pretreatments and wt% $MgCO_3$ as well as from the interactions between them. The $\delta^{18}O$ values are not directly affected by wt% $MgCO_3$ (direct effect of 0.0282 in Table S2, supporting information, $p = 0.3768$; Figure 3A), but they are most strongly affected by the interaction of Roasting and wt% $MgCO_3$ (Roasting:wt% $MgCO_3$ interaction effect of −0.2341 in Table S2, supporting information, $p = 0.0000$; Figure 3A). This same negative effect of roasting of species with higher wt% $MgCO_3$ occurs in $\delta^{13}C$ values, but it is more pronounced in $\delta^{18}O$ values (Figure 3). Both Light and Hard H_2O_2 significantly and negatively affect $\delta^{18}O$ values at

TABLE 3 Mean difference from controls of oxygen and carbon stable isotope ratios of marine organisms after pretreatment for removal of organic material. All values in Δ‰VPDB. Complete dataset is available in Table S1 (supporting information)

	Roasting		Light Bleach		Hard Bleach		Light H_2O_2		Hard H_2O_2	
Species	$\delta^{18}O$	$\delta^{13}C$	$\delta^{18}O$	$\delta^{13}C$	$\delta^{18}O$	$\delta^{13}C$	$\delta^{18}O$	$\delta^{13}C$	$\delta^{18}O$	$\delta^{13}C$
Barbatia novaezealandiae	−0.54	−0.40	−0.08	−0.04	0.15	0.07	−0.27	0.02	−0.23	0.12
Mytilus galloprovincialis	0.41	−0.71	0.16	−0.22	0.68	−0.22	0.00	−0.22	−0.01	−0.24
Galeolaria hystrix	−3.01	−1.29	−0.40	−0.21	−0.02	−0.46	−1.03	−0.57	−0.72	−0.64
Haliotis iris	−0.34	−1.07	1.08	0.15	1.52	0.15	0.71	−0.33	0.77	0.32
Arthrocardia corymbosa	−2.89	−2.54	0.15	0.96	0.75	1.00	−1.60	−0.14	−1.44	−0.16
Evechinus chloroticus (test plates)	−2.04	−0.03	0.34	0.08	0.31	−0.18	0.08	−0.08	−0.06	−0.20
Hippomenella vellicata	−1.75	−0.41	−0.07	0.06	−0.18	−0.11	−0.64	0.07	−0.69	0.16
Celleporaria emancipata	−1.23	−0.17	−0.02	−0.03	−0.09	0.11	0.16	0.14	−0.76	0.39
Evechinus chloroticus (spines)	−1.03	−0.14	0.22	0.00	0.47	0.03	0.41	0.04	0.73	0.02
Hornera foliacea	−1.55	−0.38	0.07	0.00	−0.02	−0.06	−0.23	−0.06	−0.52	0.01
Hornera robusta	−0.98	−0.43	0.24	−0.17	0.17	−0.07	−0.14	−0.23	−0.20	−0.08
Austromegabalanus psittacus	0.28	0.05	0.83	0.08	0.43	−0.05	0.12	0.01	0.46	0.00
Pecten novaezealandiae	0.63	−0.45	0.08	−0.29	0.18	−0.29	−0.29	0.02	0.34	−0.32
Elminius modestus	−0.74	−0.15	0.04	0.07	−0.02	0.05	−0.24	0.24	−0.28	0.08
Calloria inconspicua	−0.37	−0.10	−0.28	−0.15	0.08	−0.13	−0.22	−0.25	−0.35	−0.42
Minimum:	−3.01	−2.54	−0.40	−0.29	−0.18	−0.46	−1.60	−0.57	−1.44	−0.64
Mean:	−1.01	−0.55	0.16	0.02	0.30	−0.01	−0.21	−0.09	−0.20	−0.06
Maximum:	0.63	0.05	1.08	0.96	1.52	1.00	0.71	0.24	0.77	0.39
Standard deviation:	1.07	0.64	0.37	0.28	0.42	0.31	0.54	0.20	0.58	0.27

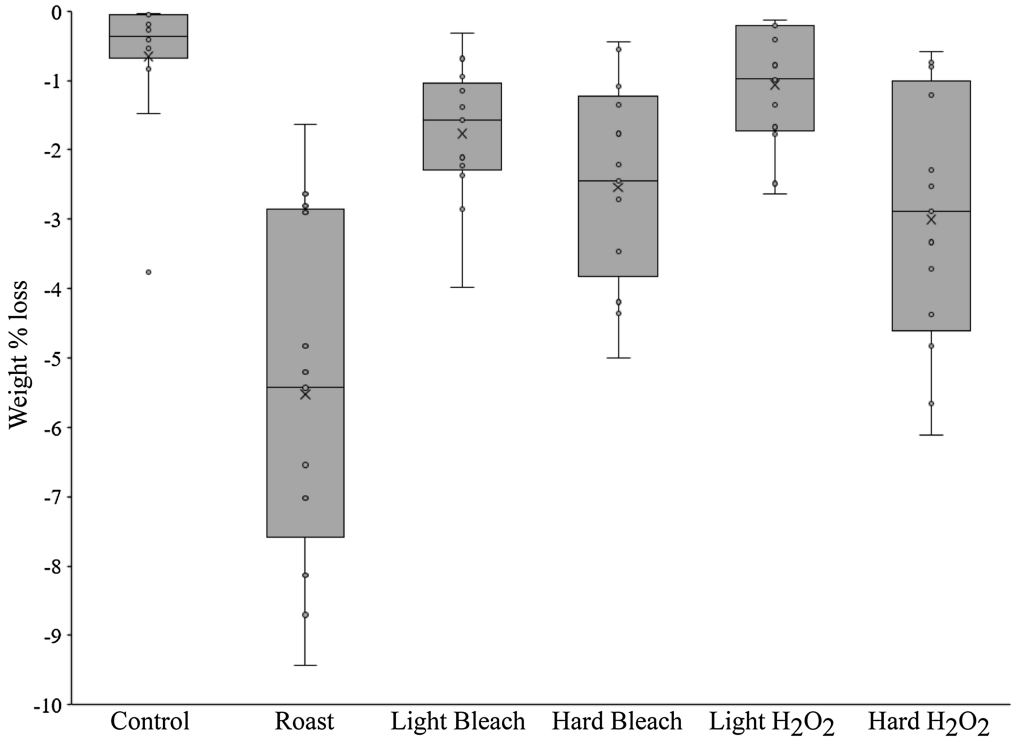

FIGURE 1 Box and whisker plot of weight percentage loss of organic material by pretreatment

TABLE 4 Mean percentage weight loss for 13 species and six pretreatments to remove organic material. Modified from Smith et al (Table 4)[72]

	Control		Roasting		Light Bleach		Hard Bleach		Light H_2O_2		Hard H_2O_2	
Species	**Mean**	**Stdev**	**Mean**	**Stdev**	**Mean**	**Stdev**	**Mean**	**Stdev**	**Mean**	**Stdev**	**Mean**	**Stdev**
Barbatia novaezealandiae	0.029	0.029	2.895	0.218	0.948	0.141	1.766	0.505	0.198	0.081	1.206	0.086
Mytilus galloprovincialis	0.047	0.024	2.810	0.120	1.141	0.111	2.208	0.144	0.158	0.023	0.805	0.120
Galeolaria hystrix	0.416	0.095	8.129	0.108	2.225	0.198	3.464	0.364	1.769	0.136	6.111	1.140
Haliotis iris	0.829	0.392	9.438	1.146	1.325	0.176	4.998	1.404	0.990	0.323	5.658	4.387
Evechinus chloroticus (test plates)	0.267	0.029	6.542	0.356	1.386	0.060	1.349	0.222	0.969	0.122	2.288	0.249
Hippomenella vellicata	1.481	0.300	8.705	0.209	3.975	0.357	4.354	0.083	2.485	0.231	4.827	0.072
Celleporaria emancipata	3.759	2.532	6.519	0.340	2.065	0.288	2.443	0.339	2.639	0.433	4.378	0.133
Hornera foliacea	0.511	0.065	5.429	0.240	2.362	0.275	3.466	0.343	1.671	0.536	3.716	0.060
Hornera robusta	0.371	0.064	4.825	0.191	1.568	0.069	2.712	0.576	0.777	0.017	2.887	0.245
Austromegabalanus psittacus	0.191	0.077	2.632	0.035	0.683	0.059	1.086	0.067	0.407	0.085	3.330	2.880
Pecten novaezelandiae	0.056	0.030	7.026	8.339	2.110	2.716	0.555	0.052	0.211	0.042	0.734	0.138
Elminius modestus	0.533	0.094	5.201	0.054	2.858	0.116	4.194	0.233	1.354	0.377	2.521	0.137
Calloria inconspicua	0.035	0.006	1.635	0.092	0.314	0.039	0.445	0.066	0.129	0.017	0.590	0.038
Mean of all species	0.656		5.522		1.766		2.542		1.058		3.004	

higher wt% $MgCO_3$ (Light H_2O_2:wt% $MgCO_3$ interaction effect in Table S2, supporting information, of −0.0720, $p = 0.0007$; Hard H_2O_2: wt% $MgCO_3$ interaction effect in Table S2, supporting information, of −0.0857, $p = 0.0000$; Figure 3A). As Light and Hard Bleach have insignificant direct effects and interactions with wt% $MgCO_3$ (Table S2, supporting information; Figure 3A), we conclude that bleaching does not significantly affect $\delta^{18}O$ values. The effect of bleaching, while not significant, affected the $\delta^{18}O$ values in the same positive direction as in the $\delta^{13}C$ values.

The $\delta^{13}C$ values were most strongly affected by wt% $MgCO_3$ (direct effect of −0.2255 in Table S3, supporting information, $p = 0.0002$; Figure 3B). There was a strong interaction between Roasting and wt% $MgCO_3$ with higher levels of wt% $MgCO_3$ producing a larger reduction in $\delta^{13}C$ values (Roasting:wt% $MgCO_3$

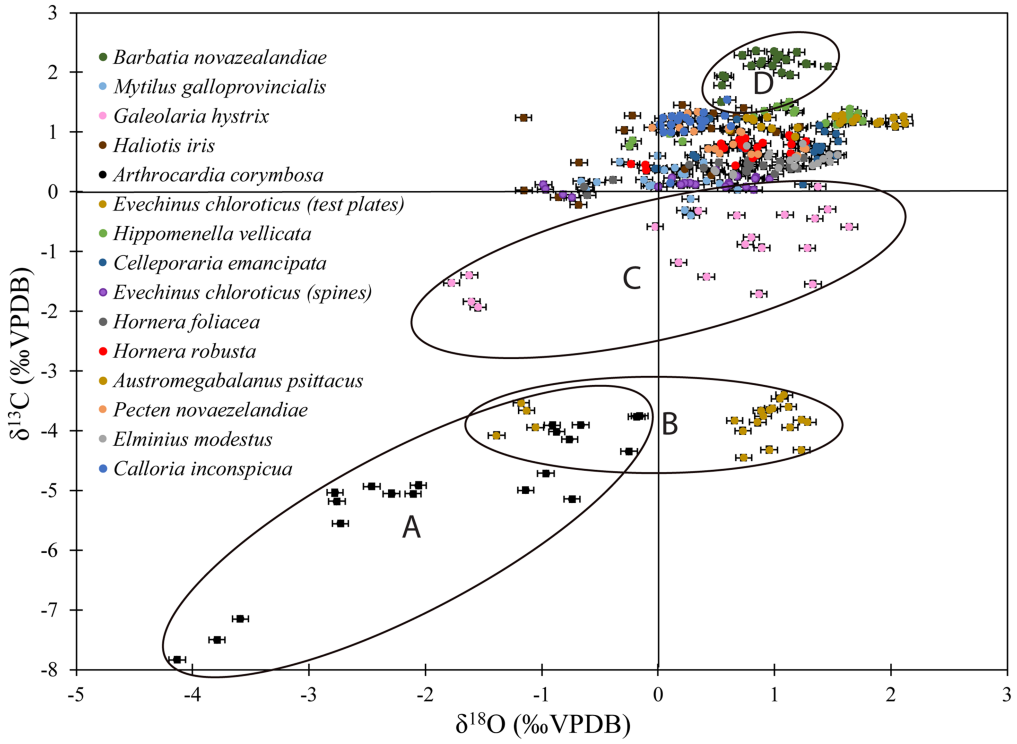

FIGURE 2 Plot of $\delta^{18}O$ and $\delta^{13}C$ values labelled by species. Error bars indicate precision of the mass spectrometer: $\delta^{18}O \pm 0.07‰$VPDB and $\delta^{13}C \pm 0.04‰$VPDB [Color figure can be viewed at wileyonlinelibrary.com]

interaction effect of −0.0975 in Table S3, supporting information, $p = 0.0000$; Figure 3B). There was also a small interactive effect between Light Bleach and wt% $MgCO_3$ with higher levels of wt% $MgCO_3$ resulting in an increase in $\delta^{13}C$ values (Light Bleach:wt% $MgCO_3$ interaction effect in Table S3, supporting information, of 0.0443, $p = 0.0131$; Figure 3B). However, any effects of bleaching are small. As Light and Hard H_2O_2 have insignificant direct effects and interactions with wt% $MgCO_3$ (Table S3, supporting information; Figure 3B), we conclude that H_2O_2 does not significantly affect $\delta^{13}C$ values.

4 | DISCUSSION

4.1 | Organic removal

Robust temperate marine shells, such as those used in this study, consist mainly of mineral $CaCO_3$ (with various amounts of $MgCO_3$), but some are additionally strengthened by layers of proteins, polysaccharides and pigments – generally lumped into the category of "organic material". Molluscan carbonate is among the most organic-rich, with 2–4% of the shell by weight being organic.[89] The weight loss reported here following pretreatment (means of 0.6 to 5.5 wt%) suggests that most if not all organic material was removed, either by combustion or molecular dissociation. Combustion resulted in the most weight loss (Table 4), suggesting that it removed skeletal material as well as organic material. This mass loss has been attributed to loss of structural water and CO_2 in bioapatite.[10,90] Pretreatment using H_2O_2 can also cause dissolution of surface carbonate,[26,30,34] which may lead to an overestimate of weight loss due to removal of organics, and can affect the isotopic ratio, especially the $\delta^{18}O$ value.[60] Bleaching appears to have been effective without removing skeletal material.

Complete removal of organic material from biogenic carbonate can, nevertheless, be difficult because organics are often finely disseminated throughout the skeleton.[18,38,73] Powdering or crushing of samples prior to pretreatment could potentially overcome this issue, although it can lead to sample loss during handling. More recent studies have been effectively using wt%N as a proxy for the amount of organic material to better quantify wt% loss of organic matter.[91] Smith et al[72] and Roberts et al[60] further discuss the literature on removal of organic material from complex skeletons.

4.2 | Mineralogy

Regardless of the pretreatment, HMC skeletons had significantly more variable and more negative isotope ratios than those lacking high-Mg calcite. It has been known since the late 1940s to early 1950s that the isotope ratios of biogenic carbonate are strongly affected by the mineralogy of the skeleton,[23] as well as environmental parameters such as water isotopic composition, temperature, and salinity,[3] kinetic effects,[92] and vital effects.[93] Regardless of the pretreatment, the species making HMC skeletons had significantly more variable and more negative isotope ratios than those lacking high-Mg calcite. Lowenstam (1961) was the first to show increasingly negative $\delta^{18}O$ values with increasing $MgCO_3$ content in biogenic

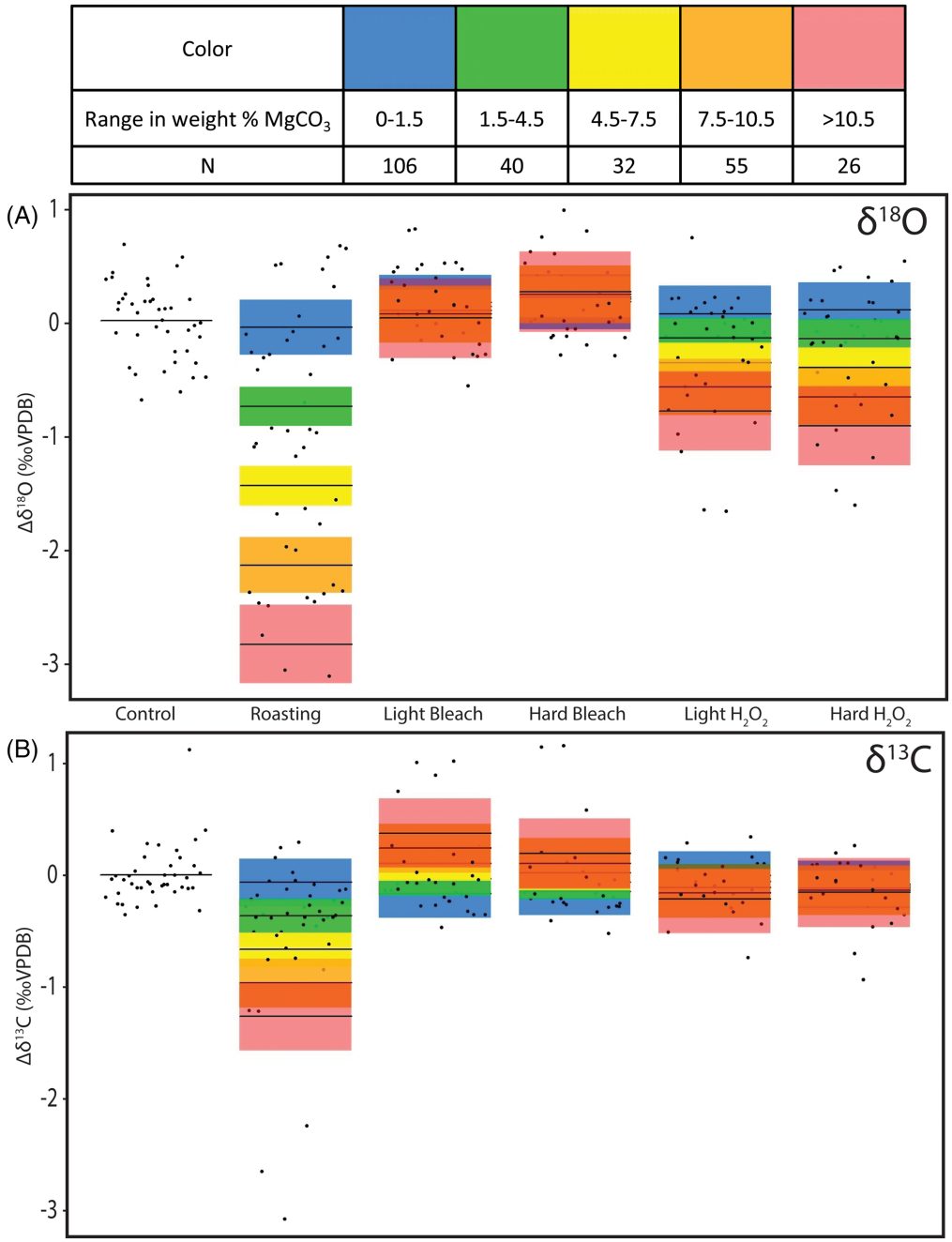

FIGURE 3 Contrast plots showing differences between the control and each pretreatment by mineralogy (i.e., weight percent $MgCO_3$) for A, $\delta^{18}O$ and B, $\delta^{13}C$ values. Dots represent the partial residuals from the statistical model. Differently colored boxes represent the different weight percent $MgCO_3$ bins. The shading of each colored box represents the 95% confidence interval around the mean differences between the controls and each pretreatment. Horizontal bars represent the means. Random x-axis jitter has been applied to data points within each treatment to prevent sample overlay to make them more visible [Color figure can be viewed at wileyonlinelibrary.com]

carbonate.[94] Substitution of atoms of lower mass in the crystal structure of minerals (e.g., Mg^{2+} vs Ca^{2+} in Mg-calcite) favors the enrichment of the isotope of greater mass in the solid (e.g., ^{18}O vs ^{16}O in the carbonate anion) due to the decreased vibrational energy of the lattice.[95] This "$MgCO_3$ effect" on oxygen isotopes has since been confirmed by subsequent studies that empirically showed an increase in the oxygen isotope fractionation factor with increasing Mg content,[77,96,97] leading to a Mg correction to paleotemperature equations based on $\delta^{18}O$ values.[97] Pretreatment leading to partial dissolution (such as roasting and H_2O_2) can cause a similar effect on $\delta^{18}O$ values, depending on how the Mg is incorporated into the calcite lattice.[98,99]

In contrast to oxygen isotopes, much less is known about the effect of Mg content in calcite on $\delta^{13}C$ values. We found a significant effect among Mg-calcitic skeletons, especially when treated by roasting. Others[100–102] have found a small increase in the carbon

isotope fractionation factor with increasing Mg content, and have argued that studies using HMC species should take into account Mg content, but that the Mg content does not significantly impact interpretations based on the $\delta^{13}C$ value of LMC below ~6 wt% $MgCO_3$ in calcite.

Isotope chemists studying mixed mineralogy skeletons, such as those we report here, are thus in the position of using one paleotemperature equation to correct for the relative amount of aragonite and calcite and another to correct for Mg content, which at the minimum will lead to a greater margin of error, and could be mutually confounding. For example, in a species such as *H. iris*, where the proportion of aragonite to calcite is naturally variable and unknowable unless measured through the destructive process of XRD, correcting the isotope ratios becomes untenable. One response has been to limit stable isotope studies to simple biominerals. Our model suggests that avoiding HMC skeletons would also assist in ensuring robust results.

4.3 | Pretreatments

On average, the pretreatments increased the variability of the isotope ratios. The standard deviation (SD) of the control for $\delta^{18}O$ values was 0.76‰VPDB and, for $\delta^{13}C$ values, 1.84‰VPDB. The average SD of the five pretreatments for $\delta^{18}O$ values was 0.86‰VPDB and, for $\delta^{13}C$ values, 1.91‰VPDB. The most variable pretreatment was roasting which had a SD of 1.20‰VPDB for $\delta^{18}O$ values and 2.21‰ VPDB for $\delta^{13}C$ values. Others have also noted the increase in the variability of $\delta^{18}O$ and $\delta^{13}C$ values in response to pretreatments.[30,52,103] The net effect of increased variability is to reduce the precision of the paleotemperature proxy.

4.3.1 | Roasting

The net effect of roasting is typically a decrease in $\delta^{18}O$ and $\delta^{13}C$ values (Table 1). Our results showed that roasting causing a significant decrease in $\delta^{18}O$ values (mean of −1.01‰) across all mineralogies (Table 3; Table S2, supporting information; Figure 3). A change in the $\delta^{18}O$ value of this magnitude results in a paleotemperature decrease of ~1°C.[97] The $\delta^{13}C$ values also decreased (mean of −0.55‰) across the mineralogical spectrum, but in general not as much as $\delta^{18}O$ values except for species with higher wt% $MgCO_3$ (Table 3; Table S3, supporting information; Figure 3). Of the previously published experiments that quantify the effect of roasting (Table 1), 42% of the studies found that it had a significant effect on $\delta^{18}O$ values (mean: −0.42‰) and 14% on $\delta^{13}C$ values (mean: −0.09‰) (Table 1). As this pretreatment has the most significant effects on stable isotope ratios, most studies now avoid roasting or do so only at or below 200°C to reduce this problem.

Roasting affects the stable isotope ratios of different minerals, and thus different taxa, in different ways. Many $CaCO_3$ skeletons are composed of the metastable polymorphic phases aragonite and HMC.[72] For example, roasting of aragonite corals typically lowers both $\delta^{18}O$ and $\delta^{13}C$ values, but the former more significantly (Table 1).[28,30] The problem arises from the fact that roasting of metastable aragonite at higher temperatures and durations causes remineralization to the more stable form of calcite.[23,28,45,72] Some organisms, especially bryozoans, precipitate a range of mineralogies, sometimes in a single colony, from aragonite to HMC to IMC to LMC.[63,64,72] Roasting can affect stable isotope ratios in the metastable $CaCO_3$ phases (Table 1). If a polymorph transition occurs from aragonite to calcite during pretreatment, isotopic exchange with organic O and C is possible.[83]

Our Roasting pretreatment did significantly alter the mineralogy of the samples as measured by the aragonite:calcite ratio and the wt% $MgCO_3$ for our specimens made of >6 wt% $MgCO_3$.[72] Roasting lowered the $\delta^{18}O$ values of all our species except the aragonite + LMC mussel *Mytilus galloprovincialis*, the LMC barnacle *Austromegabalanus psittacus*, and the LMC scallop *Pecten novaezealandiae* (Table 3). Roasting lowered the $\delta^{13}C$ values of all our species except the LMC barnacle *Austromegabalanus psittacus* (Table 3).

4.3.2 | Bleaching

Our results indicate no significant effect of bleaching on $\delta^{18}O$ values across all mineralogies (Table S2, supporting information; Figure 3). Our two bleaching pretreatments (i.e., 30% concentration for 3 h or 100% for 3 days) bracketed the average from previous studies (mean: 5.3% for 21.2 h) (Table 1). Of the previously published experiments that quantify the effect of bleaching (Table 1), only 23% found that it had a significant effect on $\delta^{18}O$ values (mean: −0.10‰) and 30% on $\delta^{13}C$ values (mean: −0.13‰). Likewise, the $\delta^{13}C$ values did not change significantly except for the Light Bleach pretreatment on the HMC species which saw a small but significant increase in $\delta^{13}C$ values (Table S3, supporting information; Figure 3). Previous studies in IMC and HMC echinoids and bryozoans, found a similar increase in $\delta^{13}C$ values.[27,41]

NaOCl at 2–3% strength typically has a pH of ~10.5 which affects both the organic and the mineral phases of biogenic carbonates.[22,104] Any observed changes in the $\delta^{18}O$ and $\delta^{13}C$ values of bleached samples may result from isotope exchange with the NaOCl solution or the replacement of $CaCO_3$ by $Ca(OH)_2$ at exposed grain surfaces.[26,105] Our Light and Hard Bleach pretreatments did not significantly alter the mineralogy of the samples as measured by the aragonite:calcite ratio and the wt% $MgCO_3$.[72] The net effect of bleaching on $\delta^{18}O$ and $\delta^{13}C$ values is typically insignificant (Table 1), further supported by our results.

4.3.3 | Oxidizing

Our results indicate a small but significant negative effect of H_2O_2 on $\delta^{18}O$ especially in HMC species (Table S2, supporting information; Figure 3). Our two H_2O_2 pretreatments (i.e., 5% concentration for 3 h

or 30% for 3 days) bracketed the average used in previous studies (mean: 23.1% for 12.7 h) (Table 1). Of the previously published studies that quantify the effect of oxidation of organic matter by H_2O_2 (Table 1 excluding Zhang et al,[34] as discussed above), only 13% of the experiments found a significant effect on $\delta^{18}O$ values (mean: 0.02‰) and 13% on $\delta^{13}C$ values (mean: −0.08‰). In contrast, $\delta^{13}C$ values have not been shown to change significantly across the mineralogical spectrum, either here (Table S3, supporting information; Figure 3) or in the literature.[56]

H_2O_2 is, unfortunately, strongly corrosive to $CaCO_3$.[105] At ~30% strength, H_2O_2 has a pH of ~5 which promotes dissolution of carbonate.[22,26,30,73,104,106] Susceptibility to dissolution increases with Mg content.[43] Weight loss during our study suggested that some dissolution did occur. Our Light and Hard H_2O_2 pretreatments did not significantly alter the mineralogy of the samples as measured by the aragonite:calcite ratio and the wt% $MgCO_3$.[72] The net effect of H_2O_2 pretreatment is dependent on its duration.[107] Our results suggest a small significant negative effect of H_2O_2 on $\delta^{18}O$ values especially in HMC species. HMC species are probably more susceptible to dissolution from the H_2O_2 as they have a higher solubility than LMC.[108] The interaction of skeletal $MgCO_3$ and pretreatment solutions such as H_2O_2 has been attributed to a combination of solution chemistry and reaction kinetics.[13,98]

4.4 | Implications for fossil specimens

This study was conducted on modern specimens, but it also has application to fossils. When considering fossil specimens, one must keep in mind the possible effect of fossil diagenesis on O and C stable isotopes.[11] This is especially true of originally aragonite, HMC, and IMC fossils whose O and C stable isotope ratios can be altered when transforming to LMC during diagenesis.[64,109] Given sufficient time and temperature, the organic content is eventually reduced in fossils as it is altered to hydrocarbons or graphite.[110,111] However, even modern biogenic carbonate is susceptible to preservational differences which can bias O and C stable isotope ratios.[59]

Oxygen stable isotope ratios are frequently used to calculate paleotemperatures.[1–3] Diagenesis as well as the effects of the various pretreatments documented in this study will affect these values. Therefore, the resulting calculated paleotemperatures will be affected. The bigger the pretreatment effect, the bigger the impact on the calculated temperatures. The negative effect on $\delta^{18}O$ values of roasting and H_2O_2 pretreatments of species with higher wt% $MgCO_3$ would be significant[98] and should be avoided.

4.5 | Standard practice

One of the main complications of the biogenic carbonate pretreatment debate is the lack of a consistent standard practice.[20,60] There are inconsistencies in the concentrations of chemical used, in the temperatures used, and in the duration of exposure time (Table 1). Previous authors have written: (1) "There is little consensus among workers on the chemical pretreatment of skeletal samples, which contain small amounts of organic material"[107]; (2) "Differences in sample preparation methods raise obvious questions about the effect of pre-measurement handling techniques on obtained $\delta^{18}O$ and $\delta^{13}C$ values"[50]; (3) "Unfortunately, no comprehensive and detailed investigations of the best techniques adapted to the removal or organic matter … have been published yet"[112]; (4) "There is no agreement on the efficiencies of organic removal of the various pretreatment methods employed"[90]; (5) "Pretreatment methods cause isotopic bias in themselves and should probably best be avoided"[113]; (6) "Despite a considerable body of literature, there is still no consensus as to which chemical treatment methods are 'the best'"[22]; and (7) "To date, many works discussed the effect of pre-treatment on the isotopic composition of the biogenic and inorganic carbonates. However, no agreement has been reached so far".[103]

One might think that a discipline-wide standard should exist. After all, the effects of pretreatment of biogenic carbonate on their O and C stable isotope ratios (Table 1), major element chemistry,[70,73,106,107,114–116] and mineralogy have been well studied.[71–73,117,118] Pretreatment methods to remove organic material have been shown to affect O and C isotope ratios in non-carbonate minerals.[119,120] NaOCl, a common and long-established chemical pretreatment used on bioapatites to remove unwanted organic material during preparation for inorganic O and C analyses, for example, involves pretreatment with sodium hypochlorite, followed by acetic acid to remove diagenetic and secondary carbonates.[31,121,122] Isotopic fractionation can result if such chemical pretreatments are not used.[22,31,90] During pretreatment, exchanges can occur with atmospheric CO_2 which may have affected our isotope ratios as others have shown experimentally.[34,123] Similarly, in organic C isotope analysis, acid washing is a common pretreatment, which has been found to significantly affect isotope ratios.[124]

The pretreatment debate is also ongoing among the users of clumped isotopes,[125,126] those working in other isotope systems,[127,128] those focused on abiogenic carbonates,[113,129,130] and those measuring stable isotopes in soft tissues.[131] On-line geochemistry list-serves reflect the lack of consensus on what, if any, pretreatment should be used. Some argue that there is no need for removal of organic matter, because organic matter does not react quickly enough with the phosphoric acid. Others argue that organic removal is still required, although it does no good or harm. This is to assure consistency with historical measurements which did pretreat as a matter of course. As a result of the confusion, some working on major element chemistry of biogenic carbonates have recommended avoiding all chemical pretreatment and those working on skeletal carbonate mineralogy have done the same.[72,106,132]

5 | CONCLUSIONS

Among under-represented taxonomic groups known for precipitating a complex variety of mineralogies, removal of organic material (never

more than 5 wt%) is most effectively achieved by roasting, but this treatment results in unwelcome impacts on the isotope ratios. Both $\delta^{18}O$ and $\delta^{13}C$ values are reduced in species with high wt% $MgCO_3$ in calcite, particularly when treated by roasting. Both H_2O_2 pretreatments significantly and negatively affect $\delta^{18}O$ values at higher wt% $MgCO_3$, whereas the bleaching pretreatment does not significantly alter $\delta^{18}O$ or $\delta^{13}C$ values. The complex interaction of skeletal carbonate mineralogy with pretreatment method shows that care must be taken when designing future experiments or interpreting past results.

On balance, we recommend the abandonment of pretreatment for removal of small amounts of organic material from biogenic skeletal carbonate of mixed mineralogies. Pretreatment can confound stable isotope ratios, whereas there is little evidence of a significant effect of small amounts of organic matter on isotope ratios. Researchers are increasingly doing the same with other skeletal groups.[26,36,48,49,52,107,133] Regardless of the amount of organic material originally present, the different pretreatments yield varying results when using samples with mixed mineralogies. If pretreatment is deemed to be necessary in your mixed mineralogically samples, we argue that roasting, especially at higher temperatures and durations, should be avoided. Use minimal bleaching (explicitly indicating the concentration and duration of exposure), and in general avoid using HMC species in O and C stable isotope studies.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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