Carbonate clumped isotope values compromised by nitrate-derived NO₂ interferent

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Abstract

Δ⁴⁷ based clumped isotope thermometry has enabled reconstruction of Earth’s surface temperatures independent of the source of oxygen within the carbonate. It has been postulated that carbonate samples can contain contaminants that cause isobaric interferences, compromising measured Δ⁴⁷ values and reconstructed temperatures. The exact nature of contaminants and isobaric interferents, however, largely remained unidentified.

Here, we demonstrate that compromised measurements can be identified through high-precision analysis of Δ⁴⁸ alongside Δ⁴⁷. We provide evidence that nitrate constitutes a serious contaminant even if present in the carbonate in sub-wt-% quantities only. During phosphoric acid digestion of carbonates at 90°C, nitrate decomposes to NO₂, which, in turn, is not effectively removed during subsequent purification of carbonate-derived CO₂ using cryogenic traps and gas chromatography (packed Porapak Q column at -15°C). In dual clumped isotope space, samples affected by variable sub-ppm contributions of NO₂ to CO₂ plot along a characteristic slope of -0.3, in agreement with theoretical predictions. Nitrate contamination occurs in a synthetic calcite precipitated using Ca(NO₃)₂, a pedogenic carbonate nodule, a plasma-ashed echinoid spine, ETH-3 (a recently assigned anchor for Δ⁴⁷ analysis of carbonates) and, presumably, in a bioapatite (Greenland shark dentine). The extent of NO₂ generation may depend on analytical setup and additional parameters such as nitrate concentration inside the carbonate, acid digestion temperature, reaction time, filament current and filament age.
Sequential bleaching tests reveal that nitrate contaminant can be effectively removed from carbonates if samples are pre-treated overnight with 3 wt-% sodium hypochlorite (NaOCl). Our high-precision long-term $\Delta_{47}$ (CDES 90) values for ETH-1 and ETH-2 (Bernecker et al., 2023) and for bleached ETH-3 exactly confirm recently assigned $\Delta_{47}$-I-CDES values for these standards. Unless independent evidence is given that NO$_2$ contamination is irrelevant for a specific analytical setup we strongly recommend that ETH-3 is bleached with 3 wt-% NaOCl overnight in order to enable accurate projection of raw data to the I-CDES. Moreover, systematic bleaching tests should be carried out on unknown samples in order to avoid any isotopic bias.

1 Introduction

Carbonate clumped isotope thermometry represents a well-established method to reconstruct carbonate formation temperature independent of the isotopic composition of the water the mineral crystallized from (Ghosh et al., 2006; Eiler et al., 2011). Its temperature dependence is expressed by the $\Delta_{47}$ value which quantifies the extent to which the internal distribution of heavy carbon and oxygen isotopes amongst carbonate isotopologues with cardinal mass 63 departs from stochastic partitioning, as predicted by the given bulk carbon and oxygen isotopic composition of the carbonate. Since excess abundance measurements of $^{13}$C-$^{18}$O isotopologues of cardinal mass 63 cannot be carried out on the carbonate directly, these measurements are performed on CO$_2$ that is extracted from the carbonate by reacting it quantitatively with phosphoric acid (Ghosh et al., 2006). Due to the relatively low average natural abundance of $^{13}$C-$^{18}$O bearing CO$_2$ isotopologues of mass 47 of ca. 45 ppm and the low mass resolution of most gas source mass spectrometers, $\Delta_{47}$ analyses can be easily compromised by non-CO$_2$ isotopologues which occur isobaric to m/z 47 (e.g., Eiler & Schauble, 2004; Huntington et al., 2009; Eagle et al., 2010; Bergmann et al., 2018). Eiler & Schauble (2004) noticed that N$_2$O, hydrocarbons and chlorinated hydrocarbons cause serious isobaric bias during $\Delta_{47}$ analyses of atmospheric CO$_2$ samples. Whereas hydrocarbons and chlorinated hydrocarbons could be effectively removed by exposing the analyte CO$_2$ to UV-radiation and cryogenic traps, the bias arising from N$_2$O contamination required an empirical correction approach taking into account additional analysis of well-defined CO$_2$-N$_2$O mixtures and N/C ratios specific of samples and mixtures. On the contrary, for carbonates subjected to phosphoric acid digestion, the origin of isobaric contamination has been poorly investigated. Identification of samples apparently unbiased by isobaric m/z contaminants relied on the application of ambiguous criteria from which it often remained open if sample-$\Delta_{47}$ was affected at all. The most widely applied is the
extent to which the excess abundance of apparent m/z 48 CO₂ isotopologues in carbonate-derived CO₂ (expressed as $\Delta_{48}$ value) deviates from that in equilibrated gas standards (Huntington et al., 2009). This approach assumes that the contaminant contributing to m/z 47 also contributes to m/z 48, an assumption that is not necessarily true. For example, SO contaminant will just cause isobaric interference on m/z 48, but not on m/z 47. Moreover, m/z 48 based approaches to identify samples with compromised $D_{47}$ values often rely on arbitrarily or statistically set threshold values for $\Delta_{48}$ (e.g., Passey et al., 2010; Snell et al., 2014; Davies & John, 2017; Staudigel et al., 2018) from which the relevance and extent of isobaric contamination on m/z 47 remains unconstrained. It has been demonstrated that secondary electrons originating from the interaction of the major m/z 44 ion-beam with the flight tube, electromagnet, or Faraday collectors, also contribute to measured m/z 47 and 48 intensities, shifting the baseline below m/z 47 and 48 Faraday cups into the negative range (He et al., 2012; Bernasconi et al., 2013; Fiebig et al., 2016). If unaccounted for, this negative pressure baseline (PBL) effect results in biases that scale inversely with the natural abundance of m/z 47 and 48 CO₂ isotopologues, such that the bias in $\Delta_{48}$ roughly exceeds that in $\Delta_{47}$ values by one order of magnitude (Wacker et al., 2016; Bernecker et al., 2023). In this respect, uncommon $\Delta_{48}$ values are not unambiguously diagnostic of isobaric interferences on m/z 47 either, especially when no PBL correction of measured raw m/z 47 and 48 intensities has been performed.

The latest generation of gas source mass spectrometers benefits from an improved setup that matches the requirements for more precise simultaneous detection of CO₂ isotopologues whose abundances vary by 5 to 6 orders of magnitude. With the introduction of the 253plus gas source mass spectrometer, the generation and collection of secondary electrons has been reduced, the signal to noise ratio of amplifiers improved and continuous monitoring of the negative PBL below m/z 47 and 48 made possible (e.g., Fiebig et al., 2019). With these improvements, long-term $\Delta_{47}$ and $\Delta_{48}$ analyses with precisions very close to mass spectrometric shot-noise level have now become available (Bernecker et al., 2023). Highest-precision dual clumped isotope thermometry allows researchers to resolve the extent to which the bulk stable and clumped isotope compositions of a pristine carbonate were affected by kinetic limitations in addition to temperature (Fiebig et al., 2019; Guo & Zhou, 2019; Guo, 2020; Bajnai et al., 2020). Moreover, temperature can be accurately isolated from the kinetic information based on measured $\Delta_{47}$ and $\Delta_{48}$ values alone (Bajnai et al., 2020; Fiebig et al., 2021; Davies et al., 2022, 2023; Tagliavento et al., 2023a). In addition, it has become possible to reconstruct peak-diagenetic temperatures from dual clumped analysis of partially altered samples (Staudigel et al., 2023a, Staudigel et al., 2023b). Here, we show that combined high-precision analysis of $\Delta_{47}$ and $\Delta_{48}$ also helps to
identify the presence and origin of isobaric contaminants in natural carbonate samples. We demonstrate that the presence of trace amounts of nitrate in a given carbonate sample leads to compromised \( \Delta_{47} \) and \( \Delta_{48} \) values. We also show that, following our methods of CO\(_2\) preparation and purification, the presence of pyrite does not affect \( \Delta_{48} \) values. Nitrate can be effectively removed from the sample by pre-treatment with 3 wt-% NaOCl overnight.

### 2 Methods

#### 2.1 Samples

Dual clumped isotope analyses were performed on pedogenic carbonates, ETH-3, a synthetic calcite, an echinoid spine, Greenland shark dentine, carbonate skeletons from brachiopods, warm- and cold-water corals, and on authigenic methane seep carbonates:

Pedogenic carbonate samples were directly prepared from untreated nodules using mortar and pestle. HGB-42a represents a pedogenic nodule from Hell Gate Basin located in the Miocene Barstow Formation, Mud Hills, California, USA (Loughney & Badgley, 2017). Samples HGB-42a\(_{\text{NEW}}\) and HGB-42a\(_{\text{NEWEST}}\) represent newly prepared aliquots from the same nodule.

BW-33.7 is a calcite that has been precipitated at a temperature of 33.7°C through slow and dropwise addition of a Ca(NO\(_3\))\(_2\) solution to a NaHCO\(_3\) solution (Spahr et al., 2021). Analytical-grade calcium nitrate tetrahydrate Ca(NO\(_3\))\(_2\) \* 4 H\(_2\)O (99.95 % purity, Merck KGaA, Darmstadt, Germany) and sodium bicarbonate NaHCO\(_3\) (≥ 99.7 % purity, Merck KGaA, Darmstadt, Germany) were dissolved separately in bi-distilled water in two 5-liter laboratory bottles, to obtain solutions with a concentration of 10 mmol/L. Both glass vessels were sealed gas-tight and connected to each other by a transfer tube for the solution and a compensation line. Afterwards, the glass bottles were equilibrated in an air thermostat while the solutions were continuously stirred. After reaching the target temperature of 33.7°C in the solutions, they were kept for seven days in the thermostat before the precipitation was started in order to achieve full isotopic equilibration between dissolved inorganic carbon species and water. Temperature was controlled using a Julabo LC6 temperature controller with a temperature stability of ±0.03°C. Precipitation was initiated by slowly (≈ 10 mL/h) adding the calcium nitrate solution to the sodium bicarbonate solution using a VWR PP1080 peristaltic pump while magnetic stirring. Precipitated CaCO\(_3\) was separated from its parent solution by vacuum filtration. Raman analysis revealed that the precipitated CaCO\(_3\) was pure calcite (Fig. 1).

Sample SD-MAI-S has been made available from spines of *Stronglyocentrotus Droebachiensis*. This echinoid specimen has been hatched in an aquaculture facility at the University of Maine at a temperature of 8.5°C. Spines collected from this specimen have been ground to powder
using mortar and pestle. An aliquot of the ground and homogenized powder (SD-MAI-S2) was treated by low temperature plasma ashing under oxygen at a flow rate of 400 ml/min for at least 30 minutes in order to remove organic matter as CO$_2$ (Davies & John, 2018).

Samples LP-SM (*Desmophyllum Pertusum*), A4 (*Acropora cervicornis*) and A6 (*Acropora sp.*) are cold- and warm-water corals, respectively. These aragonitic samples were described in more detail in Davies et al. (2022). KASU_NOT and KIWIINC are calcitic eggshells of a Cassowary and a Kiwi, respectively (Tagliavento et al., 2023a). MV130b and MBHB represent new aliquots from Brachiopod shells of species *Magellania venosa* and *Hemithiris psittacea*, respectively, previously sampled for dual clumped isotope investigations by Davies et al. (2023). Carb2A (aragonite) and Carb3C (calcite) (DIVE ID’s 54-2 and 61-2, respectively) are authigenic methane seep carbonates from Formosa Ridge previously described and analyzed for their dual clumped isotope composition by Staudigel et al. (2024).

ETH-3 represents Cretaceous chalk from the Isle of Rügen, Germany, which is – along with ETH-1 and ETH-2 – used as a low-temperature anchor for standardization of mass spectrometric $\Delta^{17}$ raw data obtained on carbonate unknowns (Bernasconi et al., 2021).

Finally, we also analyzed carbonate-bearing dentine from a Greenland Shark (sample SLGDenA). This sample was prepared as described in Löffler et al. (2019).

### 2.2 Bleaching of samples

With the exception of SD-MAI-S, SD-MAI-S2 and SLGDenA, bleached aliquots (suffix OXI) were prepared from each sample and analyzed along with their unbleached aliquots. Samples were treated with 3 wt-% sodium-hypochlorite (NaOCl) and left to react overnight (at least 8 hours). Afterwards, the samples were rinsed by adding demineralized water, thoroughly mixing the solution and sample, centrifuging and decanting the solution. This rinsing step was repeated 4 times in total. The samples were subsequently dried in a vacuum oven at 30°C.

Given aliquots of HGB-42a and ETH-3 were treated twice with 3 wt-% NaOCl (suffix OXII) in order to test the effect of sequential bleaching steps on the bulk and clumped isotopic composition. In addition, a new aliquot of ETH-3 was bleached once again (suffix NEW, OXI) in order to test the reproducibility of bleaching and its effect on the isotopic composition. From HGB-42a a third aliquot (suffix RINSE) was treated exactly the same way as described above, but with de-ionized water instead of 3 wt-% NaOCl.

### 2.3 Dual clumped isotope analysis

Replicate aliquots corresponding to 10 mg of pure CaCO$_3$ were weighed into silver capsules and stored in a vacuum oven at 30°C until analysis. For isotopic analysis, the capsules were
loaded into the low-blank autosampler of the automated gas preparation line HAL, which is
connected to the dual inlet system of a 253plus gas source mass spectrometer (Fiebig et al.,
2019). With the exception of SLGDenA, which was reacted for 90 minutes, all samples were
digested with 108-109 wt-% phosphoric acid for 30 minutes. Some samples were additionally
reacted in quartz capsules (suffix QZ). Whenever samples were reacted in quartz capsules no
silver capsules were present in the acid. The evolved CO$_2$ was purified using four cryogenic
traps (-80°C) and gas chromatography (packed Porapak Q column, 80-100 mesh, at -15°C), as
detailed in Fiebig et al. (2019). The purified CO$_2$ was finally analyzed for its dual clumped
isotope composition at an m/z 44 intensity of 16 Volts in 13 acquisitions consisting of 10 cycles
each (20 seconds integration time per cycle). Mass spectrometric m/z 47-49 raw intensities
were corrected for a negative PBL effect using a m/z 47.5 half mass cup, and $\Delta_{47}$ and $\Delta_{48}$ values
are finally reported on the CDES 90. Preparation of equilibrated gases, PBL correction and data
normalization has been described in detail by Bernecker et al. (2023). Unless indicated
otherwise, all PBL corrected data was evaluated in the pooled approach using D47crunch
(Daëron, 2021), considering variance minimization of anchors and samples over all sessions
for the determination of session-specific correction parameters a, b and c (MSP approach,
Bernecker et al., 2023). Treating data this way allows for a better correction of drifts that may
occur between and within single sessions, e.g., through temporal variations in the phosphoric
acid reaction environment (Daëron, 2021). However, as we show in Section 4.1, our analytical
setup yields $\Delta_{47}$ and $\Delta_{48}$ values for ETH-3 that are compromised by the presence of isobaric
non-CO$_2$ interferent. Since ETH-3 makes up a significant fraction of all measured data (ca. 8
%), carbonate anchor ETH-3 was, therefore, excluded from variance minimization in the MSP
approach. This can be easily achieved providing individual identifiers for each unbleached
ETH-3 replicate. Since bleached ETH-3 aliquots were not suffering from contamination
anymore (see Section 4.3), it was pooled over all bleached replicates in this approach.

In the last session, we tested the effect of reaction time and sequential NaOCl pre-treatment on
the dual clumped isotope composition of ETH-3. In addition to the MSP approach described
above, this session was evaluated separately in two more modes: 1) treating unbleached ETH-
3 (ETH-3), once bleached ETH-3 (ETH-3$_{OXI}$), twice bleached ETH-3 (ETH-3$_{OXI2}$), newly
bleached ETH-3 (ETH-3$_{NEW,OXI}$) and unbleached ETH-3 reacted for 10 minutes (ETH-3$_{10min}$)
as individual samples, while anchoring all raw data against nominal values of equilibrated gases
both for $\Delta_{47}$, $\Delta_{48}$ and $\Delta_{49}$ (Bernecker et al., 2023) (see Section 4.3); 2) pooling over all bleached
ETH-3 replicates (n = 34) while anchoring raw data against nominal values of equilibrated
gases and ETH-1 and ETH-2 for $\Delta_{17}$ exclusively (see Section 4.8). Again, individual identifiers were used for each unbleached ETH-3 replicate in these two additional approaches.

Mass spectrometric shot noise limit (total counting time of 2600 seconds per replicate at an m/z 44 intensity of 16 Volts) is 7.0 ppm and 23.4 ppm for $\Delta_{17}$ and $\Delta_{18}$, respectively.

D47crunch also provides the option to normalize $\delta^{13}C_{VPDB}$ and $\delta^{18}O_{VPDB}$ data of unknown samples relative to ETH carbonate standards (Daëron, 2021). For this purpose, we have considered the nominal $\delta^{13}C_{VPDB}$ and $\delta^{18}O_{VPDB}$ values of ETH-1 and ETH-2 reported by Bernasconi et al. (2018).

### 2.4 Raman spectroscopy

Raman measurements were carried out with a custom set-up in Frankfurt described in detail elsewhere (Bayarjargal et al., 2018). We used an OXXIUS S.A. Laser-Boxx LMX532 laser ($\lambda = 532$ nm) and a spectrograph (Princeton Instruments ACTON SpectraPro 2300i) equipped with a Pixis256E CCD camera. Measurements were performed in reflection geometry with the polarized laser light. The spectral resolution of our spectrometer is 3 cm$^{-1}$ and the accuracy is around 0.5 cm$^{-1}$. Raman measurements were performed with an exposure time of 5-30 sec and with a power of 8-26 mW. Raman spectra were acquired on three samples: (I) a transparent large single calcite crystal, (II) BW-33.7 and (III) SD-MAI-S2.

![Raman spectra](image)

**Fig. 1.** Raman spectra of samples I (single calcite crystal), II (BW-33.7) and III (SD-MAI-S2). For further discussion see Section 4.2.
2.5 Modeling of isotopic bias introduced by isobaric interferents

The presence of contaminants being isobaric to CO₂ will bias δ¹³C, δ¹⁸O, Δ₁₇ and Δ₄₈ values of carbonate-derived CO₂ along vectors which are characteristic of the chemical and isotopic composition of the contaminant. Supplementary Table S1 presents an excel sheet (“isobaric interference calculator”) that computes how isobaric contamination with SO⁺, N₂O⁺, NO₂⁺, CH₃S⁺, CS⁺ and CCl⁺ will affect δ¹³C, δ¹⁸O, Δ₁₇ and Δ₄₈ values of CO₂. This calculator considers H, N, S and Cl isotopic compositions of interfering gases relative to VSMOW, V-Air-N₂, VCDT and SMOC respectively, whereas C and O isotope compositions of analyte CO₂ and interferents are provided relative to the VPDB-CO₂ scale. In a first step, the abundances of m/z 44-49 isotopologues in the mixture made up of CO₂ and interferent are calculated assuming random distribution of all isotopes amongst interfering isotopologues. In a next step, apparent δ¹³C-CO₂ and δ¹⁸O-CO₂ values are calculated from 45/44 and 46/44 abundance ratios characteristic of the mixture, as outlined for pure CO₂ by Brand et al. (2010). In a final step, apparent Δ₁₇, Δ₄₈ and Δ₄₉ values of the mixture are calculated from corresponding 47/44, 48/44 and 49/44 isotopologue ratios and apparent stochastic values computed from apparent δ¹³C-CO₂ and δ¹⁸O-CO₂ values.

3 Results

3.1 Contamination vectors in Δ₁₇ - Δ₄₈ and Δ₄₈ - Δ₄₉ space

Δ₁₇ - Δ₄₈ vectors theoretically predicted for relatively small contributions of NO₂, N₂O, SO, CCl, CS and CH₃S to analyte CO₂ are shown in Figure 2a, assuming the analyte CO₂ has bulk and clumped isotopic composition corresponding to VPDB-CO₂ and stochastically predicted values of zero, respectively. It can be easily shown using our isobaric interference calculator (Supplementary Table S1) that the clumped isotope composition of the analyte CO₂ has no significant effect on the extent of interferent-specific isotopic bias. For interferents without m/z 44-46 isotopologues (SO, CH₃S, CCl) contamination vectors represent linear mixing lines over the entire range of relative contributions of analyte CO₂ and interferent. For NO₂ and CS the slope of the mixing vector remains constant within three digits (and, hence, can be approached linearly) if these interferents make up less than 1 ppm (NO₂) and 100 ppm (CS) of the total gas mixture. Δ₁₇ and Δ₄₈ values are very sensitive to contamination from NO₂. A contribution of 40 ppb of NO₂ to CO₂ would already result in an isotopic bias of -6 ppm and 19 ppm in Δ₁₇ and Δ₄₈, respectively, closely corresponding to the average lower and upper boundary of our fully
propagated 2 SE for $\Delta_{47}$ and $\Delta_{48}$ analysis. This bias increases to -72 ppm in $\Delta_{47}$ and to 240 ppm in $\Delta_{48}$, if the molar fractional abundance of NO$_2$ increases to 500 ppb. Within this range, the slope of the corresponding contaminant vector is -0.30, and relatively insensitive to natural variations in the abundances of C, N and O isotopes in the contaminant NO$_2$ and analyte CO$_2$.

For example, if, within the above range of NO$_2$ contribution, $\delta^5$N and $\delta^{18}$O are varied by $\pm$50‰, this will only make the slope of the vector change by $\leq \pm$0.01 and $\pm$0.04, respectively. An additional variation of $\pm$0.04 in this slope can be introduced through carbon and oxygen isotope variations in the analyte CO$_2$. Of all contaminants investigated in this study, NO$_2$ is the only one that introduces a paired $-\Delta_{47}/+\Delta_{48}$ bias.
Fig. 2. Biases ($\Delta(A_i)$) in the carbonate clumped isotope composition introduced by the presence of trace amounts of NO$_2$, N$_2$O, CH$_3$S, CS, CCl and SO in analyte CO$_2$. A: $\Delta_47$ vs. $\Delta_48$; B: $\Delta_{49}$ vs. $\Delta_{48}$. Arrows represent contamination vectors with H, C, N, O, S and Cl isotope compositions of interfering molecules corresponding to VSMOW, VPDB-CO$_2$, V-Air-N$_2$, VPDB-CO$_2$, VCDT and SMOC, respectively, and analyte CO$_2$ isotope compositions corresponding to VPDB-CO$_2$ and stochastic distribution of isotopes. Numbers in A and B provide contribution of interferent to CO$_2$ pool in ppm. Inset in A presents a zoom-up of A showing isobaric biases on a scale corresponding to the 2 SE of $\Delta_47$ and $\Delta_48$ measurements of ±6 ppm and ±20 ppm, respectively, that are typically obtained for 8 replicate measurements. For further discussion see Section 3.1.

Relative to NO$_2$, $\Delta_47$ and $\Delta_48$ values are less sensitive to contamination by N$_2$O, with $\Delta_47$ being much more affected than $\Delta_48$. The bias in $\Delta_47$ becomes comparable to the upper limit of our analytical $\Delta_47$ precision of ±6 ppm if the molar fraction of N$_2$O reaches 35 ppm. If this fraction increases to 5000 ppm, the bias in $\Delta_47$ reaches 856 ppm, while the corresponding $\Delta_48$ bias (-3 ppm) still remains well below its detection limit. Within this range, N$_2$O contamination would be indicated by a near-vertical displacement of measured $\Delta_47$ and $\Delta_48$, relative to unbiased values. A significant bias in $\Delta_48$ is only introduced if the $\delta^{18}$O of N$_2$O contaminant differs from that of the analyte CO$_2$. For example, if the contaminant $\delta^{18}$O deviates by $\mp$50‰ from the analyte CO$_2$, biases of ±50 ppm in $\Delta_48$ are obtained for an addition of 1000 ppm N$_2$O, finally reducing the slope of the N$_2$O contaminant vector from near-infinity to values of 3.6 and -3.3, respectively. Contamination with CCl fragment also affects $\Delta_47$ much more than $\Delta_48$. Over the range of natural variations in $\delta^{13}$C and $\delta^{37}$Cl values, contamination by CCl is indicated by a slope of ~8 in $\Delta_47$ vs $\Delta_48$ space, with both $\Delta_47$ vs $\Delta_48$ being positively biased. A similarly steep slope of ~4.6, on the contrary, would be indicative of CH$_3$S contamination. Presence of SO would be indicated by a horizontal vector in dual clumped isotope space, since mass 48 represents the lightest SO isotopologue. SO contamination would become detectable in $\Delta_48$ if its molar fraction in the CO$_2$ analyte pool makes up $\geq$ 0.1 ppb. For example, the presence of 0.1 ppb SO would introduce a bias of 20 ppm in $\Delta_48$, and this bias scales linearly with the molar fraction of SO. Though CS does not have m/z 48 isotopologues, it affects $\Delta_48$ through the stochastic composition much more than $\Delta_47$, resulting in a slightly positive slope of ~0.04. Of all contaminants investigated in this study, it is the only one that introduces negative biases both in $\Delta_47$ and $\Delta_48$ values.

Correlations between $\Delta_48$ and $\Delta_49$ could also be used to obtain further constraints on the nature and presence of contaminants (Fig. 2b). This should work most effectively for CCl, which introduces a bias in $\Delta_49$ that is ca. 2500 and 300 times larger than that in $\Delta_48$ and $\Delta_47$, respectively, largely independent of the isotopic composition of the contaminant gas and analyte CO$_2$. Biases due to CH$_3$S occur magnified in $\Delta_49$ by factors of ~200 and ~40 relative to the
corresponding biases in $\Delta_{48}$ and $\Delta_{47}$, respectively. For CS and SO, the bias in $\Delta_{49}$ represents
roughly 100 % (slope ~1) and 75 % (slope ~1.35) of the bias observed in $\Delta_{48}$, again largely
independent of natural variations in the abundances of C, S and O isotopes. NO$_2$ bias in $\Delta_{49}$ is
inversely correlated with the (positive) bias in $\Delta_{48}$. Over the natural range of isotope variations
in NO$_2$ and CO$_2$, its absolute value, however, only makes up 10-50 % of the $\Delta_{48}$ bias.
Considering that the 2 SE of $\Delta_{49}$ measurements based on 8 replicate measurements can reach
±1000 ppm under most stable analytical conditions (Bernecker et al., 2023), isobaric
contamination of CO$_2$ with NO$_2$ and SO becomes detectable by means of $\Delta_{49}$ at relatively high
levels of contamination only, i.e., when the bias in $\Delta_{48}$ becomes > 1 % (Fig. 2b) such that $\Delta_{48}$
values well outside the natural range would be obtained. Although N$_2$O has no m/z 49
isotopologues, $\Delta_{49}$ becomes sensitive to N$_2$O contamination via its effects on the stochastic
composition at relatively high concentrations of several thousands of ppm.

3.2 Dual clumped isotope analysis

Final sample data pooling over all sessions is provided in Table 1, together with fully
propagated errors (2 SE), which also take into account uncertainties associated with data
normalization. PBL corrected $\delta^{55}$-$\delta^{99}$ data of all replicates (input data for D47crunch) is listed
in Supplementary Table S2.
The last session in which ETH-3 bleaching tests were performed was also evaluated
individually in two more modes (see Sections 2.3, 4.3 and 4.8). Corresponding sample data is
listed in Supplementary Tables S3 and S4.
**Table 1.** Bulk and dual clumped isotope composition of CO₂ evolved from phosphoric acid digestion of investigated samples. δ¹³C, δ¹⁸O and Δ values are reported in ‰-deviation relative to V-PDB, V-SMOW and stochastic clumping. Number of replicates is indicated by n. Suffixes “G”, “V”, “c”, “CLEAN”, “bleach” and “OXI” indicate samples pretreated with 3 wt-% NaOCl.

### 3.3 Raman Spectroscopy

Four characteristic Raman modes of calcite can be clearly identified in the Raman spectra of the transparent large single calcite crystal (I), BW-33.7 (II) and SD-MAI-S2 (III) at around...
1086, 711, 281 and 156 cm$^{-1}$ (Fig. 1). We also observed a weak peak at 1066 cm$^{-1}$ close to the symmetrical stretching mode of CO$_3$ groups at 1086 cm$^{-1}$ in samples I and II.

4 Discussion

4.1 Isobaric contamination from NO$_2$

Experimentally precipitated calcite using Ca(NO$_3$)$_2$

The synthetic calcite that was precipitated from an equilibrated DIC pool using Ca(NO$_3$)$_2$ (BW-33.7) exhibits a significant $\Delta_{47}/\Delta_{48}$ disequilibrium offset with respect to its formation temperature of 33.7°C, irrespective if it was reacted in a quartz (BW-33.7$_{QZ}$) or in a silver capsule (BW-33.7) (Fig. 3a). The bleached aliquot reacted in a quartz capsule (BW-33.7$_{OXI, QZ}$) plots indistinguishable from the dual clumped isotope composition predicted for that sample according to the equilibrium $\Delta_{47} - \Delta_{48} - 1/T$ relationship of Fiebig et al. (2021). The observed disequilibrium offset in the untreated sample describes a slope of $-0.26 \pm 0.18$ that is consistent with the slope of -0.30 characteristic of NO$_2$ contamination (Fig. 3a).

Pedogenic carbonate HGB-42a

The dual clumped isotope compositions of the different aliquots of sample HGB-42a are displayed in Figure 3b. The two bleached pedogenic carbonate aliquots, reacted in silver (HGB-42a$_{NEWEST, OXI}$) and quartz capsule (HGB-42a$_{NEWEST, OXI, QZ}$), respectively, exhibit $\Delta_{47}$ and $\Delta_{48}$ values that are indistinguishable from equilibrium. On the contrary, all non-bleached aliquots from the same nodule, either reacted in quartz or silver capsules, depart significantly from equilibrium, plotting below the equilibrium line. Of these, the aliquot exposed to water (HGB-42a$_{NEWEST, RINSE}$) exclusively shows the smallest disequilibrium offset. Error-weighted linear regression over all aliquots yields a slope of $-0.26 \pm 0.06$ which – within its uncertainty – corresponds to the slope theoretically predicted for NO$_2$ contamination of -0.30 (Fig. 3b).

Spicule SD-MAI-S

The untreated spicule reacted in a silver capsule (SD-MAI-S) plots slightly above the $\Delta_{47}/\Delta_{48}$ equilibrium line (Fig. 3c). Its $\Delta_{47}$ value indicates a precipitation temperature that is significantly lower than the true temperature of formation of 8.5°C. Accordingly, the dual clumped isotope composition of this sample may have been affected by CO$_2$ absorption kinetics. The plasma-ashed aliquots reacted in a silver (SD-MAI-S2) and in a quartz capsule (SD-MAI-S2$_{QZ}$), on the contrary, plot significantly below the equilibrium line, and the corresponding $\Delta_{47}$ values now indicate a formation temperature that is significantly higher than 8.5°C. Again, the observed
differences in the dual clumped isotope compositions between these three samples would be in agreement with SD-MAI-S2 being affected by NO2 contamination (Fig. 3c).

Fig. 3. Dual clumped isotope composition of unbleached and bleached aliquots of BW-33.7 (A) and HGB-42a (B), and of untreated (SD-MAI-S) and plasma-ashed (SD-MAI-S2) spicule (C). Error bars display fully propagated 2 SE (95% confidence interval level). Solid line represents OGLS regression through sample-specific data points (Daëron & Vermeesch, 2024).
Although we exclusively use CO₂ equilibrated at 1000°C and 25°C, respectively, for the projection of mass spectrometric raw data to the CDES 90, we have regularly run ETH-1, ETH-2 and ETH-3 (reacted in silver capsules) along with unknown carbonate samples. Recently, we documented that our long-term average Δ₁⁷ values for ETH-1 and ETH-2 confirmed the internationally agreed upon Δ₁⁷ values of 0.2052 ± 0.0016 ‰ (n = 232) and 0.2085 ± 0.0015 ‰ (n = 215), respectively, within their corresponding 1 SE uncertainties. Our long-term Δ₁⁷ value for ETH-3, however, occurred 10 ppm lower than its assigned value of 0.6132 ± 0.0014 ‰ (Bernecker et al., 2023). Interestingly, session mean values for ETH-3 show a significant (p-value < 0.005) correlation between Δ₁⁷ and Δ₄₈ values (Fig. 4d). The error-weighted linear regression slope of -0.31 ± 0.15 agrees very well with that characteristic of NO₂ contamination.

On the contrary, any significant correlations between session’s mean Δ₁⁷ and Δ₄₈ values are not displayed for Carrara, ETH-1, ETH-2 and GU1 (Fig. 4a-c, e).

Fig. 4. Session-mean Δ₁⁷ and Δ₄₈ values of unbleached aliquots of Carrara marble (A), ETH-1 (B), ETH-2 (C), ETH-3 (D) and GU1 (E). Error bars display 2 SE (95 % confidence interval level) based on long-term repeatability. Pearson tests reveal that only ETH-3 (D) displays a significant correlation (p < 0.05) between Δ₁⁷ and Δ₄₈ values. Solid line (D) represents OGLS regression through all data points (Daëron & Vermeesch, 2024).

4.2 Origin of NO₂ interferent
The extent of NO$_2$ bias in $\Delta_{47}$ and $\Delta_{48}$ values of non-bleached aliquots of BW-33.7 (Fig. 3a), HGB-42a (Fig. 3b), SD-MAI-S2 (Fig. 3c) and ETH-3 (Fig. 4d) likely does not depend much on the choice of capsule material. Non-bleached BW-33.7 reacted in quartz capsules has $\Delta_{47}$ and $\Delta_{48}$ indistinguishable from those of the same sample reacted in silver capsules (Table 1, Fig. 3a). The spicule aliquot reacted in a quartz capsule (SD-MAI-S2$_{QZ}$) has a significantly lower $\Delta_{48}$ value than the aliquot reacted in silver (SD-MAI-S2), but was analyzed in two replicates only (Table 1, Fig. 3c).

BW-33.7 was precipitated using a Ca(NO$_3$)$_2$ solution. Traces of nitrate might, therefore, still be present within this sample. It has been well demonstrated by Hu et al. (2019) that nitrate decomposes to NO$_2$ in the presence of phosphoric acid and in the absence of any silver. These authors reacted 2 mg of calcite doped with 1 mg of KNO$_3$ at 72°C in a McCrea reaction vessel with 100% H$_3$PO$_4$. They acquired Raman spectra on the produced gas and noticed a mode at 752 cm$^{-1}$ that is characteristic of NO$_2$. They also extracted CO$_2$ from mixtures of 100-200 µg of CaCO$_3$ and ~50µg of KNO$_3$ using the Gas Bench II setup, where the mixture was reacted with 6-8 drops of phosphoric acid at 72°C for 4 h in borosilicate Exetainer topped with butyl rubber septum. Although the CO$_2$ was passed in a He carrier gas over a PoraPlot Q column (25 mm, 0.32 mm o.d.) at 70°C before being introduced into the gas source mass spectrometer, they noticed an abnormal increase of the m/z 46 background, indicating the presence of $^{14}$N$^{16}$O$^{16}$O in the eluted CO$_2$ (Hu et al., 2019). These authors proposed the following reactions to be responsible for NO$_2$ production from nitrate:

$$NO_3^- + H^+ \rightarrow HNO_3$$  \hspace{1cm} (1)

$$4 HNO_3 \rightarrow 4 NO_2 + O_2 + 2 H_2O$$  \hspace{1cm} (2)

We have investigated BW-33.7 and SD-MAI-S2 using Raman spectroscopy. Up to now, no study has been reported about a possible substitution of NO$_3$ groups with CO$_3$ groups in calcite, which was detected by Raman spectroscopy. In the case of a possible incorporation of NO$_3$ groups in calcite, we are expecting a shift, broadening of Raman modes, or the presence of a characteristic mode of NO$_3$ groups as a weak peak. According to the literature, the characteristic mode of NO$_3$ is located in the range of 1040-1069 cm$^{-1}$ in different nitrates (Liu et al., 2008; Hetmańczyk et al., 2009; Bishop et al., 2021). Most of them are in the range of 1043-1056 cm$^{-1}$, especially in calcium nitrates. Liu et. al. (2008) used Raman spectrometry to study the hygroscopic behavior of individual Ca(NO$_3$)$_2$ and internally mixed Ca(NO$_3$)$_2$/CaCO$_3$ particles. They identified NO$_3$ groups based on the symmetrical stretching mode $v_1$. An isolated free NO$_3$ ion has this characteristic mode at 1049 cm$^{-1}$, which shifts from 1050 cm$^{-1}$ to 1056 cm$^{-1}$ with decreasing humidity in the presence of Ca$^{2+}$ in Ca(NO$_3$)$_2$. We also observed a weak peak at
1066 cm$^{-1}$ close to the symmetrical stretching mode of CO$_3^-$ groups at 1086 cm$^{-1}$ in samples I and II. Our measurements reveal a detection limit of 0.7 - 0.5 % for this peak in these two samples. For sample SD-MAI-S2 (III) the detection limit was > 1 %, probably explaining why this peak is not detected in this sample. Though we cannot entirely exclude it, we do not think this peak is indicative of nitrate, either present in calcite as Ca(NO$_3$)$_2$ or substituted for CO$_3^{2-}$. First, this peak is well outside the range of 1043 cm$^{-1}$ to 1056 cm$^{-1}$ that is typical for Ca(NO$_3$)$_2$, which has been used to prepare sample BW 33.7. Second, we do not detect any shift and broadening of all Raman modes of calcite structure which would be expected if nitrate substitutes for carbonate. Instead, we propose its occurrence is best explained by the presence of small amounts of ikaite (CaCO$_3\cdot$6H$_2$O) (Sánchez-Pastor et al., 2016) which may have formed on the surface of the calcite through the absorption of moisture. Note that the presence of only 350 ppb of NO$_2$ in the analyte CO$_2$ pool is required to explain the observed bias in $\Delta$$_{47}$ and $\Delta$$_{48}$ in BW-33.7 relative to the expected equilibrium composition. This is well below the detection limit of nitrate of 1000 ppm characteristic of spontaneous Raman spectroscopy (Diaz & Hahn, 2020; Schlack et al., 2021).

Spicule samples SD-MAI-S is composed of magnesium calcite. Spicules usually contain 0.1 % (w/w) organic matrix which is mainly composed of proteins and glycoproteins (e.g., Ameye et al., 1999). Proteins, in general, can contain up to 20 % of reduced nitrogen (Krul et al., 2019). Apparently, reduced nitrogen in the organic matrix is oxidized to nitrate when the powdered spicule is treated with molecular oxygen in the plasma asher. As for BW-33.7, the presence of only 350 ppb of NO$_2$ in the reacted CO$_2$ is required to explain the isotopic bias observed in SD-MAI-S2 relative to the untreated SD-MAI-S (Fig. 3c). Considering SD-MAI-S to be unbiased, as suggested by its position above the equilibrium line characteristic of CO$_2$ absorption, we postulate that reduced nitrogen in organic matter needs not be removed from carbonate for accurate dual clumped isotope analysis.

Trace amounts of nitrate, as indicated by NO$_2$ bias in $\Delta$$_{47}$ and $\Delta$$_{48}$ values of non-bleached aliquots, even seem to be present in pedogenic carbonate sample HGB-42a and ETH-3. ETH-3 is composed of Cretaceous chalk which consists of carbonate skeletons of marine phytoplankton and micritic carbonate. It has a non-carbonate content of less than 2 % (Neumann, 2012). Phytoplankton cells utilize seawater nitrate as their nitrogen source (e.g., Kim et al., 2022). Traces of nitrate, therefore, might still be contained in the organic remnants within ETH-3.

4.3 Removing nitrate contaminant from carbonates
All bleached aliquots of BW-33.7 and HGB-42a have in common that they exhibit significantly higher $\Delta_{47}$ and lower $\Delta_{48}$ values than their unbleached counterparts, demonstrating that nitrate contamination and NO$_2$ interference is significantly suppressed in bleached aliquots (Figs. 3a, b). Overnight treatment of samples with distilled water also reduces isobaric bias in $\Delta_{47}$ and $\Delta_{48}$, however, this alone is not sufficient to quantitatively remove the NO$_2$ interference, as demonstrated by the observation that HGB-42$_{\text{NEWEST,RINSE}}$ still plots significantly below the equilibrium line, whereas the two bleached aliquots of the same sample (HGB-42$_{\text{NEWEST,OXI2}}$ and HGB-42$_{\text{NEWEST,OXI,OZ}}$) plot indistinguishable from the equilibrium line (Fig. 3b). We postulate that the nitrate contaminant in this sample is contained in organic matter and is most effectively dissolved after quantitative oxidation of organic matter to CO$_2$.

In order to test in more detail to what extent ETH-3 is affected by nitrate contamination and if the contaminant can be effectively removed through pre-treatment with 3 wt-% NaOCl we performed a sequential bleaching test and analyzed unbleached, once- and twice bleached aliquots of ETH-3 within the same session. In this session, the repeatability of $\Delta_{49}$ measurements was excellent (1100 ppm) so that we also projected $\Delta_{49}$ data to the CDES 90. Once (ETH-3$_{\text{OXI1}}$, ETH-3$_{\text{NEW,OXI1}}$) and twice bleached (ETH-3$_{\text{OXI2}}$) aliquots of ETH-3 all have indistinguishable $\Delta_{47}$ and $\Delta_{48}$ values and center around the equilibrium line (Fig. 5a). On the contrary, the two unbleached aliquots came out with lower $\Delta_{47}$ and higher $\Delta_{48}$ values than their bleached counterparts. As already observed for session mean values of unbleached ETH-3 aliquots (Fig. 4d), the slope of $-0.23 \pm 0.11$ for the error-weighted linear regression line through all data points is consistent again with NO$_2$ contamination (Fig. 5a). The correlation between $\Delta_{48}$ and $\Delta_{49}$ values independently supports the contention of NO$_2$ contamination in untreated ETH-3 (Fig. 5b). Whereas unbleached aliquots exhibit significantly higher $\Delta_{48}$ values than bleached aliquots, $\Delta_{49}$ values for bleached and unbleached aliquots of ETH-3 are within errors indistinguishable from each other – as is predicted for sub-ppm levels of NO$_2$ contamination (Fig. 2b; Fig. 5b).
Fig. 5. Dual clumped isotope composition of unbleached and bleached aliquots of ETH-3, analyzed alternatingly within a single session. Error bars display fully propagated 2 SE (95% confidence interval level). (A) $\Delta_{47}$ vs $\Delta_{48}$. Solid line represents OGLS regression through all data points (Daëron & Vermeesch, 2024); dotted line represents position of calcite equilibrium according to Fiebig et al. (2021). (B) $\Delta_{48}$ vs $\Delta_{49}$. Dotted line represents position of calcite equilibrium according to Fiebig et al. (2021) for $\Delta_{48}$ and Bernecker et al. (2023) for $\Delta_{49}$.

Three observations imply that a single bleaching step with 3 wt-% NaOCl is sufficient to effectively remove nitrate contaminant:
i) The once-bleached aliquot of BW-33.7 (BW-33.7OXI, QZ) plots indistinguishable from the equilibrium line, and its $\Delta_{47}$ value corresponds within errors to its formation temperature of 33.7°C (Fig. 3a).

ii) Once (ETH-3OXI, ETH-3NEW, OXI) and twice-bleached ETH-3 (ETH-3OXI2), all reacted in silver capsules, yield indistinguishable $\Delta_{47}$ and $\Delta_{48}$ values, centering around the equilibrium line at a temperature of 18°C (Fig. 5a).

iii) A sequential bleaching test was also performed on pedogenic carbonate sample HGB-42a. HGB-42aNEWEST, OXI2, which was bleached twice, has $\Delta_{47}$ and $\Delta_{48}$ values identical to HGB-42a-NEWEST, OXI, QZ, which was just oxidized once (Fig. 3b).

Our results demonstrate that systematic bleaching tests should be performed on unknown samples in order to exclude potential bias through isobaric contamination. Tagliavento et al. (2023a) showed that bleached aliquots of bird eggshells (Kiwi, Cassowary) reproduced $\Delta_{47}$ and $\Delta_{48}$ values of unbleached aliquots, demonstrating that the excess $\Delta_{48}$ characteristic of all bird eggshells does not arise from isobaric interferences. Staudigel et al. (2024) bleached two of their authigenic methane seep carbonates, both of which covered the entire range of observed $\Delta_{47}$ and $\Delta_{48}$ values. Again, bleached aliquots confirmed $\Delta_{47}$ and $\Delta_{48}$ values of unbleached aliquots within errors, providing independent evidence that the observed trend in dual clumped isotope data in authigenic carbonates is real. For the study of Davies et al. (2022) on corals, bleached aliquots were run along with their unbleached counterparts for two warm-water coral samples (A4, A6). Since no significant differences were obtained between bleached and non-bleached aliquots, average $\Delta_{47}$ and $\Delta_{48}$ values were pooled over all replicates in that study, without making bleached and unbleached aliquots discernable.

A comprehensive comparison of dual clumped isotope data between bleached and non-bleached aliquots from a diverse range of natural carbonates is provided in Figure 6. In addition to the data discussed above, we further considered cold-water coral LP-SM for this comparison. Of all cold-water corals analyzed by Davies et al. (2022), LP-SM showed the largest departures in
$\Delta_{47}$ and $\Delta_{48}$ from expected equilibrium values. We also prepared new homogenized sample powder from Brachiopod specimen MV130b and MBHB, originally analyzed by Davies et al. (2023). These two specimens were selected as they cover the entire disequilibrium $\Delta_{47}$ and $\Delta_{48}$ range observed by Davies et al. (2023). In all cases, $\Delta_{47}$ and $\Delta_{48}$ values of bleached aliquots reproduce those obtained on non-bleached aliquots (Fig. 6). These results strongly imply that the carbonates analyzed in the studies of Davies et al. (2022, 2023), Tagliavento et al. (2023a) and Staudigel et al. (2024) were not suffering from nitrate contamination.

**Fig. 6.** (a) Comparison of dual clumped isotope compositions of unbleached (open symbols) and bleached (closed symbols) aliquots of carbonate powder acquired from Brachiopod shells, warm- and cold-water coral skeletons, eggshells and authigenic methane seep carbonates. Inset (b) shows the long-term $\Delta_{47}$ and $\Delta_{48}$ mean values of Carrara marble and Carrara marble doped with pyrite. Error bars display fully propagated 2 SE (95 % confidence interval level). Dotted line represents the position of calcite equilibrium (Fiebig et al., 2021).

**4.5 Effects of NaOCl pre-treatment on the bulk isotope composition of carbonates**

NO$_2$ isotopologues are isobaric to CO$_2$ on m/z 46-49. NO$_2$-related $\Delta_{48}$ excesses of +200 ppm and +70 ppm, as are characteristic of HGB-42a and BW-33.7, respectively, should correspond...
with biases in δ¹⁸O of 0.1 ‰ and 0.04 ‰, respectively, whereas δ³⁴C should not be affected at all. This is largely consistent with measured bulk isotope compositions of unbleached and bleached aliquots of BW-33.7 and HGB-42 (Table 1). δ³⁴C of unbleached (-4.09 ± 0.01 ‰, reacted in QZ, and -4.09 ± 0.02 ‰, reacted in silver) and bleached aliquots of BW-33.7 (-4.11 ± 0.01 ‰, reacted in QZ) are identical, whereas δ¹⁸O of the bleached aliquot (26.59 ± 0.02 ‰) occurs slightly lower than the 26.64 ± 0.03 ‰ and 26.72 ± 0.07 ‰ obtained for unbleached aliquots reacted in quartz and silver, respectively. In case of HGB-42, the δ³⁴C value of -2.77 ± 0.01 ‰ obtained for the unbleached aliquot (reacted in quartz) is hardly distinguishable from the -2.83 ± 0.01 ‰ (reacted in quartz) and the -2.80 ± 0.01 ‰ (reacted in silver) obtained for the once and twice-bleached aliquot, respectively. The observation that the δ³⁴C of the twice-bleached sample moves closer again to that of the unbleached one may imply that the parental powder from which these three aliquots were prepared might have not been perfectly homogenized. The same is suggested by δ¹⁸O data. While a value of 29.49 ± 0.03 ‰ is obtained for the unbleached aliquot, the once and twice bleached aliquots yielded values of 29.59 ± 0.06 ‰ and 29.47 ± 0.04 ‰, respectively. Differences in δ³⁴C and δ¹⁸O between unbleached, once- and twice-bleached aliquots of ETH-3 are within errors insignificant (Supplementary Table S3). While for most samples δ³⁴C and δ¹⁸O values of treated and untreated aliquots agree well within external repeatabilities (1 SD), there are also some inconsistencies (Table 1): For the bleached Cassowary eggshell, δ¹⁸O is 0.4 ‰ lower than for the unbleached aliquot, while δ³⁴C is reproduced within uncertainties. On the contrary, δ³⁴C values of both bleached aliquots of Brachiopod shells are shifted by -1 ‰ relative to unbleached aliquots, while δ¹⁸O values between the two differ by only -0.2 ‰. Whenever prevalent, the observed shifts in bulk isotopic compositions are not unidirectional but seem to depend on the investigated archive. δ³⁴C and δ¹⁸O values of bleached cold-water coral aragonite exceed those of the untreated aliquot by 0.8 ‰ and 0.4 ‰, respectively. Pre-treatment of warm-water coral aragonite, on the contrary, does not go along with any significant shifts in the bulk isotopic compositions (Table 1).

It has been investigated in several studies which is the best and most effective way to remove organic matter from carbonates prior to isotopic analysis. Gaffey & Bronnimann (1993) determined that 2.5 wt-% NaOCl is more effective than 30 wt-% H₂O₂. While recommendations in favor of H₂O₂ have been made (Keatings et al., 2006; Wierzbowski et al., 2007), a most recent comprehensive study demonstrated that H₂O₂ pretreatment can affect the bulk isotopic composition of carbonates (δ³⁴C plus δ¹⁸O) by up to 4 ‰, and may even affect Δ₁₇ (Zhang et al., 2020). As summarized by the latter authors, several explanations have been offered for the
observed changes amongst which are the removal of organic matter, partial dissolution of carbonate, precipitation of Ca(OH)$_2$, isotopic exchange with the reagent, or the combination of all of these processes. Combined absolute changes in $\delta^{13}$C and $\delta^{18}$O accompanied with NaOCl pre-treatment applied in this study were < 1.5 ‰. However, even when significant changes in $\delta^{13}$C and $\delta^{18}$O were observed, $\Delta_{47}$ and $\Delta_{48}$ values of bleached aliquots reproduced those of unbleached aliquots within uncertainty (Table 1). We recommend interpreting $\delta^{13}$C and $\delta^{18}$O values of untreated aliquots for samples whose bulk isotopic compositions are significantly altered during NaOCl pre-treatment, i.e., whenever measured changes in $\delta^{13}$C and $\delta^{18}$O between treated and untreated aliquots exceed those predicted by our isobaric interference calculator for the observed concurrent changes in $\Delta_{47}$ and $\Delta_{48}$ values.

4.6 Isobaric contamination arising from SO

It has been postulated that reduced sulfur-bearing compounds may evoke isobaric interferences on m/z 48 and 49 through formation of SO (Guo & Eiler, 2007). Reduced sulfur compounds like pyrite are present as accessory minerals in authigenic carbonate deposits resulting from sulfate-driven anaerobic oxidation of methane. In order to investigate if the presence of pyrite in carbonates biases $\Delta_{48}$ under conditions specific for our analytical setup, internal Carrara carbonate standard was doped with 2 wt-% FeS, and this mixture subjected to phosphoric acid digestion at 90°C for 30 minutes (Staudigel et al., 2024). $\Delta_{47}$ and $\Delta_{48}$ values of the extracted CO$_2$ remained unchanged relative to the long-term averages obtained for Carrara-derived CO$_2$ (Table 1; Fig. 6b). This result demonstrates that the presence of pyrite as accessory mineral does not compromise carbonate $\Delta_{48}$ values, at least under the analytical conditions specific for HAL.

4.7 The effect of variance minimization of ETH-3 on $\Delta_{47}$ (CDES 90) and $\Delta_{48}$ (CDES 90) values presented in previous studies

The D47crunch module of Daëron (2021) that we use for data processing of PBL corrected $\delta^{45}$-$\delta^{49}$ values, considers variance minimization of $\Delta_{47}$ and $\Delta_{48}$ values of anchors and unknown samples for the determination of session-specific correction parameters a, b and c. In our case, (unbleached) ETH-3 made up a significant fraction (8 % on average) of the total number of analyzed replicates per session. Since observed variances in $\Delta_{47}$ and $\Delta_{48}$ values of ETH-3 are affected by the extent of isobaric NO$_2$ contamination, it needs to be tested if and to what extent $\Delta_{47}$ (CDES 90) and $\Delta_{48}$ (CDES 90) values of unknowns reported in previous studies were biased by variance minimization of ETH-3. For this exercise, we have re-evaluated $\Delta_{47}$ and $\Delta_{48}$ values
of calcites closely reflecting isotopic equilibrium (Fiebig et al., 2021), aragonitic skeletons of cold- and warm-water corals (Davies et al., 2022), calcitic eggshells (Tagliavento et al., 2023a), calcitic Brachiopod shells (Davies et al., 2023) and authigenic methane seep carbonates (Staudigel et al., 2024) in this study, not considering ETH-3 for variance minimization. A comparison between previously published and reprocessed data demonstrates that variance minimization of ETH-3 has not significantly affected our previously published dual clumped isotope data such that the $\Delta_{47}$-$1/T$ and $\Delta_{48}$-$1/T$ equilibrium relationships of Fiebig et al. (2021) and all interpretations made in the aforementioned publications remain valid:

Fig. 7. Comparison of reprocessed $\Delta_{47}$-$1/T$ (a) and $\Delta_{48}$-$1/T$ (b) relationships with those originally determined by Fiebig et al. (2021). In order to express the temperature dependence of $\Delta_{47}$ and $\Delta_{48}$ values, fourth order polynomials perfectly describing the theoretical temperature dependence of $\Delta_{47}$ and $\Delta_{48}$ (Hill et al., 2014), respectively, were linearly adjusted to measured data by scaling factors and a constant term, considering error-weighted least squares regression (Fiebig et al., 2021). Relationships originally determined by Fiebig et al. (2021) are well within the 95 % and 68 % confidence intervals of reprocessed
relationships. Over the entire temperature range, the difference ($\Delta$) between reprocessed and original calibrations is < 1 ppm for $\Delta_{47}$ and < 4 ppm for $\Delta_{48}$.

1) Over a temperature range of $8^\circ$ – $1100^\circ$C, the re-evaluated $\Delta_{47}$-1/T and $\Delta_{48}$-1/T equilibrium relationships are

$$
\Delta_{47} \text{ (CDES 90) (‰)} = 1.038 \left(-5.897 \frac{1}{T} - 3.521 \times 10^3 \frac{1}{T^2} + 2.391 \times 10^7 \frac{1}{T^3} - 3.541 \times 10^9 \frac{1}{T^4}\right) + 0.1848
$$
$$
\Delta_{48} \text{ (CDES 90) (‰)} = 1.038 \left(6.002 \frac{1}{T} - 1.299 \times 10^4 \frac{1}{T^2} + 8.996 \times 10^6 \frac{1}{T^3} - 7.423 \times 10^8 \frac{1}{T^4}\right) + 0.1214
$$

At any temperature, equilibrium $\Delta_{47}$ and $\Delta_{48}$ values predicted by these re-evaluated relationships differ by less than 1 ppm and 4 ppm, respectively, from the original equations provided by Fiebig et al. (2021). Re-processed calibrations are, therefore, well within the corresponding 95% confidence intervals of the $\Delta_{47}$-1/T and $\Delta_{48}$-1/T regressions provided by Fiebig et al. (2021) (Figs. 7a, b)

2) Reprocessed disequilibrium $\Delta_{47}$ and $\Delta_{48}$ values for corals are compared to those published in Davies et al. (2022) in Figure 8. The reprocessed slope for the correction of cold-water coral data is -0.80 ± 0.04, and, hence, within its uncertainty still consistent with both the model-predicted and empirical slope of -0.78 of Davies et al. (2022).

Fig. 8. Extent of disequilibrium (relative to $\Delta_i$-values predicted by independently known growth temperatures and $\Delta_{47}$-1/T and $\Delta_{48}$-1/T equilibrium relationships of Fiebig et al. (2021)) recorded in the dual clumped isotope composition of carbonate skeletons from cold-and warm-water corals. Reprocessed and original disequilibrium offsets are indistinguishable from each other. For the cold-water corals, simple linear regression through reprocessed data and the origin yields a slope of -0.80 ± 0.04 that is – within errors – indistinguishable from both the empirical (-0.78 ± 0.05) and model-predicted slopes (-0.78) obtained by Davies et al. (2022). The reprocessed disequilibrium slope for the warm-water coral dataset is also indistinguishable from the original one (-0.45 ± 0.04 compared to -0.40 ± 0.03, Davies et al., 2022).
3) Troodon and reptile eggshell data still plots indistinguishable from the equilibrium line, whereas the group of birds exhibits significant disequilibrium $\Delta_{48}$ offsets (Fig. 9).

4) The revised relationship between disequilibrium $\Delta_{47}$ and $\Delta_{48}$ in Brachiopod calcite is indistinguishable from the one published by Davies et al. (2023), confirming that a diffusional limitation is required in addition to unbalanced de(hydration)/(de)hydroxylation rates in order to explain the data (Fig. 10).

5) The empirical slope by which authigenic methane seep carbonates depart from equilibrium is indistinguishable from the one published by Staudigel et al. (2024) (Fig. 11). Within errors, back-extrapolated temperature agrees well with measured vent temperature of 3.5°C.

**Fig. 9.** Dual clumped isotope composition of eggshells, relative to the position of calcite equilibrium. Comparison of reprocessed data with original data published by Tagliavento et al. (2023a). All reprocessed data is indistinguishable from originally published data.
Fig. 10. Extent of disequilibrium (relative to $\Delta$-values predicted by independently known growth temperatures and $\Delta_47$-$1/T$ and $\Delta_48$-$1/T$ equilibrium relationships of Fiebig et al. (2021)) recorded in the dual clumped isotope composition of Brachiopod shells. Reprocessed and original disequilibrium offsets are indistinguishable from each other, and so are the corresponding simple linear regressions.

Fig. 11. Dual clumped isotope composition of authigenic methane seep carbonates, relative to the position of calcite equilibrium. Comparison of reprocessed data with original data published by Staudigel et al. (2024). Reprocessed dual clumped isotope data, slope of linear regression through reprocessed data and corresponding extrapolated vent temperature are indistinguishable from originally published data/values.
4.8 Implications for other analytical setups and the I-CDES

Fiebig et al. (2021) observed that I-CDES values (anchored relative to ETH-1, ETH-2 and ETH-3) for calcites precipitated closest to equilibrium were significantly higher than the corresponding CDES 90 values (anchored against equilibrated gases). The observed difference scaled with the $\Delta_47$ value of the sample and disappeared when ETH-3 was not considered to correct for scale compression. Moreover, their $\Delta_47$ (CDES 90) -T calibration for calcite was indistinguishable from the I-CDES calibrations obtained by Jautzy et al. (2020) and Anderson et al. (2021). The $\Delta_47$-I-CDES-T relationship of Anderson et al. (2021) integrates data over the calibrations of Breitenbach et al. (2018), Jautzy et al. (2020), Meinicke et al. (2020), and Peral et al. (2018). The calibrations of Jautzy et al. (2020), Anderson et al. (2021) and Meinicke et al. (2020) were made using the Kiel IV and the NU Carb which perform acid digestion at a temperature of 70°C under dropwise addition of phosphoric acid, instead of the 90°C characteristic of the common acid bath setup used by Fiebig et al. (2021). Notably, $\Delta_47$ (I-CDES) data of Laghetto Basso and Devils Hole calcites made at LSCE using the analytical setup of Peral et al. (2018) agreed with corresponding CDES 90 values made on the same sample material at Goethe University (GU) only if ETH-3 was not considered for the correction of the GU mass spectrometric raw data (Fiebig et al., 2021). GU and LSCE use a very similar methodology (common acid bath, acid digestion at 90°C, GC purification over packed Porapak Q), but the reaction time at GU is set to 30 minutes instead of the 15 minutes used at LSCE (Peral et al., 2018).

In order to test the effect of reaction time for our analytical setup we also reacted unbleached ETH-3 for 10 minutes (ETH-3$_{10\text{min}}$) within the last session. The dual clumped isotope composition of the extracted CO$_2$ is not significantly different from that of unbleached ETH-3 reacted for 30 minutes and nicely aligns along a slope indicative of NO$_2$ contamination if all data on bleached and unbleached ETH-3 from this session is considered (Fig. 5a). This suggests that reaction time alone is not the sole driving force for the observed NO$_2$ bias. Notably, least biased $\Delta_47$ (CDES 90) values on unbleached ETH-3 were obtained in the first two sessions. In these sessions, the filament current was set to 1.5 mA. It was only afterwards that the current was raised to 2.0 mA in order to improve sensitivity. At 2.0 mA, the largest biases in session-mean $\Delta_47$ (CDES 90) values were obtained for sessions in which the filament blew (Bernecker et al., 2023). It remains unclear how filament current may play a role. Since electron density alone is not expected to change the ionization efficiency of NO$_2$ relative to CO$_2$, it might be that the higher temperature of the filament favors preferred ionization of NO$_2$ over CO$_2$. 
In any case, the experiments of Hu et al. (2019) demonstrated that NO\textsubscript{2} formation also proceeds at 70°C. Tagliavento et al. (2023b) noted that Greenland shark dentine, analyzed in 43 replicates, departed by -30 ppm from its predicted equilibrium $\Delta_{47}$ value when it was reacted with the Kiel IV setup at 70°C for 30 minutes, using a static Porapak trap at -40°C for gas purification and fixing filament current at 2.0 mA. We also reacted Greenland shark dentine SLGDenA for 90 minutes at a temperature of 90°C using HAL. The obtained data point plots well below the equilibrium line (Fig. 12). Relative to the equilibrium $\Delta_{47}$ and $\Delta_{48}$ values predicted by the expected body temperature of 1° ± 2°C (Löffler et al., 2019), measured values have an offset of -50 ppm and 165 ppm, respectively, indicating that the Greenland shark dentine might suffer from nitrate contamination (Fig. 12). If so, the $\Delta_{47}$ bias of -30 ppm bias obtained by Tagliavento et al. (2023b) may point to the presence of significant amounts of NO\textsubscript{2} interferent when reacting at 70°C using the Kiel IV setup.

Fig. 12. The dual clumped isotope composition of Greenland shark dentine reacted at 90°C for 90 minutes plots well below the reprocessed calcite equilibrium line. Back-extrapolation of data along a slope of -0.3 yields an apparent body temperature of 0.2°C, in agreement with the range of -1.2 to 3.4°C indicated by logger data (Löffler et al., 2019).

These results imply that the extent of NO\textsubscript{2} generation in the presence (of trace amounts) of nitrate may depend on a combination of several factors amongst which are the concentration of nitrate contaminant, trap design, acid temperature, reaction time and filament current/temperature. We recommend that each lab tests their own analytical setup for the effectiveness of NO\textsubscript{2} removal, performing sequential bleaching tests on unknown samples with 3 wt-% of NaOCl. The observations of Fiebig et al. (2021) that i) $\Delta_{47}$ (CDES 90) values of Laghetto Basso and Devils Hole calcites analyzed at GU are consistent with corresponding I-CDES values made at LSCE and ii) the $\Delta_{47}$ (CDES 90) – 1/T calibration of Fiebig et al. (2021) is identical to the I-CDES calibration of Anderson et al. (2021), may imply that NO\textsubscript{2} contamination of ETH-3 is only significant in the analytical setup at GU. However, it also needs to be considered that the long-term drift in session-mean $\Delta_{47}$ (CDES 90) values at GU is on the
order of -10 to -15 ppm only (Fig. 4d), while the long-term repeatability of $\Delta_{47}$ measurements is smaller (8 – 9 ppm; Bernecker et al., 2023; Fiebig et al., 2021). Considering that the long-term repeatability of the $\Delta_{47}$ data of Anderson et al. (2021) is 36 ppm, we cannot entirely exclude a potential negative bias in ETH 3-$\Delta_{47}$ values went unnoticed in their study, i.e., that it is pure coincidence that the two calibrations are consistent.

Interestingly, our long-term $\Delta_{47}$ (CDES 90) values for ETH-1 and ETH-2 confirmed within 1 SE recently assigned $\Delta_{47}$-I-CDES values for these standards (Bernecker et al., 2023). We have also demonstrated in Figure 5a that once- and twice-bleached samples of ETH-3 yielded consistent $\Delta_{47}$ (CDES 90) and $\Delta_{48}$ (CDES 90) values. We can, therefore, pool over all 34 bleached aliquots of ETH-3 to determine a mean $\Delta_{47}$ (CDES 90) value for bleached ETH-3. Pooling over all bleached replicates has been performed in two ways: 1) Anchoring all data relative to equilibrated gases and considering all sessions for variance minimization; 2) Considering the last session exclusively and anchoring raw $\Delta_{47}$ data of all bleached ETH-3 aliquots to the nominal I-CDES values of ETH-1 and ETH-2 and nominal $\Delta_{47}$ values of equilibrated gases (to account for scale compression). These two approaches yield consistent $\Delta_{47}$ (CDES 90) values of $0.6140 \pm 0.0023$‰ (Table 1) and $0.6134 \pm 0.0021$‰ (Supplementary Table S4), respectively, which agree with the recently assigned $\Delta_{47}$-I-CDES value of $0.6132 \pm 0.0014$‰ ($n = 264$) (all errors represent 1 SE). As a consequence, ETH-3 can easily be replaced by bleached ETH-3 in those labs, in which the analytical setup does not fully suppress NO2 generation from nitrate and/or enable its quantitative removal.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\Delta_{47,\text{CDES90}}$</th>
<th>1 SE</th>
<th>$\Delta_{48,\text{CDES90}}$</th>
<th>1 SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETH-1</td>
<td>0.2052</td>
<td>0.0016</td>
<td>0.1277</td>
<td>0.0015</td>
</tr>
<tr>
<td>ETH-2</td>
<td>0.2085</td>
<td>0.0015</td>
<td>0.1299</td>
<td>0.0016</td>
</tr>
<tr>
<td>ETH-3$\text{OXI}$</td>
<td>0.6132</td>
<td>0.0014</td>
<td>0.2481</td>
<td>0.0071</td>
</tr>
<tr>
<td>GU1</td>
<td>0.2254</td>
<td>0.0008</td>
<td>-0.3998</td>
<td>0.0043</td>
</tr>
</tbody>
</table>

Table 2. Mean $\Delta_{47,\text{CDES90}}$ and $\Delta_{48,\text{CDES90}}$ obtained for ETH-1, ETH-2, ETH-3 and GU1 in Bernasconi et al. (2021) (values in bold, nominal I-CDES values) and this study. $\Delta_{47,\text{CDES90}}$ values of Bernasconi et al. (2021) for ETH-1, ETH-2 and ETH-3 were confirmed by Bernecker et al. (2023) (for ETH-1, ETH-2) and in this study (for ETH-3$\text{OXI}$, Table 1).

Since our long-term $\Delta_{47}$ (CDES 90) values for ETH anchors exactly confirm $\Delta_{47}$-I-CDES assigned for these anchors, use of our long-term values $\Delta_{48}$ (CDES 90) values for ETH-1, ETH-2, bleached ETH-3 and GU1 (Table 2) alongside $\Delta_{47}$ I-CDES for ETH-1, ETH-2 and ETH-3 might be suitable for reproducible projection of mass spectrometric raw data into dual clumped isotope space. An interlaboratory comparison is necessary to check if reproducibility on unknown samples is achieved based on the $\Delta_{48}$ (CDES 90) values listed in Table 2.
5 Conclusions

ETH-3, pedogenic carbonates and carbonate slowly precipitated from an equilibrated DIC solution using Ca(NO$_3$)$_2$ all exhibit excesses in $\Delta$$_{48}$ and depletions in $\Delta$$_{47}$ relative to corresponding aliquots bleached with 3 wt-% NaOCl. The same $\Delta$$_{48}$ enrichment/$\Delta$$_{47}$ depletion patterns are displayed by plasma-ashed echinoderm spicules relative to non-plasma ashed aliquots. The observed deviations follow a unique slope that is characteristic of interferences arising from the presence of sub-ppm quantities of NO$_2$ isotopologues in the extracted CO$_2$. The choice of acidification capsules (quartz vs. silver) has no effect on the extent of NO$_2$ generation. These results support previous findings that NO$_2$ is generated through the thermal decomposition of HNO$_3$ which, in turn, is derived from the protonation of NO$_3^-$. Apparently, in our analytical setup, NO$_2$ is not effectively removed with cryogenic traps and gas chromatography (packed Porapak Q column, 80-100 mesh, 120 cm, -15°C). We demonstrate that NO$_2$ interference arising from nitrate becomes insignificant if samples are pre-treated with 3 wt-% NaOCl overnight. The extent of NO$_2$ generation may depend on analytical setup (e.g., use of static traps vs GC, type of GC column, GC and trap temperatures, etc.) and several other factors such as nitrate concentration inside the carbonate, acid digestion temperature, reaction time, phosphoric acid concentration, filament current and filament age. We, therefore, propose that each lab individually tests the extent of NO$_2$ generation and effectiveness of NO$_2$ removal specific to their analytical setup. If NO$_2$ is determined to have a significant effect in a given laboratory, unknown carbonate samples and ETH-3 should be bleached with 3 wt-% NaOCl in order to avoid any bias in measured $\Delta$$_{47}$ and $\Delta$$_{48}$ values. Because the $\Delta$$_{47}$ (CDES 90) value of bleached ETH-3 agrees with its recently assigned I-CDES value, ETH-3 can be directly replaced by bleached aliquots. With our analytical setup and methodology, nitrate contamination and isobaric NO$_2$ bias are measurable in pedogenic carbonates, chalk, and, presumably, in bioapatites. Whereas carbon monosulfid (CS) would represent a potent isobaric interferent, we do not have any evidence that the presence of pyrite in carbonate samples compromises $\Delta$$_{48}$ analysis of extracted CO$_2$.

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and isotopic composition


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