

“Vital effects” in the ^{13}C - ^{18}O clumped isotopic composition of marine macro-invertebrates: exploring biologically mediated disequilibrium effects in echinoids and cephalopods

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Declaration of Originality

I declare that all work in this thesis is my own, unless otherwise stated or referenced.

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ABSTRACT

The stable oxygen isotopic composition of marine carbonate has historically been widely used for paleoclimate reconstruction (Emiliani 1955; Zachos 2001). This technique however applies an *a priori* assumption of $\delta^{18}\text{O}$ of the seawater. Furthermore, while the stable oxygen isotopic composition of some taxa faithfully reflects both the temperature and the $\delta^{18}\text{O}_{\text{seawater}}$ other taxa show specific discrepancies known as “vital effects” (Epstein et al. 1951).

The carbonate clumped isotope paleothermometer examines thermodynamically dependent “clumping” of ^{13}C and ^{18}O within the carbonate crystal lattice. This allows calculation of carbonate formation temperature independent of the $\delta^{18}\text{O}$ of seawater (Ghosh et al. 2006; Eiler 2007). Initially inorganic and biogenic calibrations of the clumped isotope thermometer appeared reasonably congruent (Eiler 2011, Ghosh et al. 2006; Tripathi *et al.* 2010a). Further studies however revealed vital effects in the clumped isotopic composition of corals (Thiagarajan et al. 2011; Saenger et al. 2012) with potential vital effects indicated in molluscs (Henkes et al. 2013).

In this study we assess how robust the clumped isotope thermometer is to biologically mediated non-equilibrium precipitation (vital effects) by (1) measuring the clumped isotope composition of the previously uninvestigated echinoderm group and (2) verifying and further quantifying offsets in the clumped isotope composition of modern cephalopod molluscs. This aim necessitated further refinements CO_2 purification for clumped isotope analysis, also presented within this thesis. The results of this thesis indicate that non-equilibrium signatures in the clumped isotope composition of biogenic materials are more common than initially assumed (Eiler, 2011). Furthermore the manifestation of vital effects does not occur by a single mechanism, but varies dependent on biomineralization pathway. This has significant implications for the way in which the clumped isotope composition of biogenic carbonate is used in paleoclimate reconstruction. We can no longer unreservedly assume equilibrium precipitation in the ^{13}C - ^{18}O composition of biogenic carbonate necessitating the creation of group specific calibrations using modern genera prior to the use of previously unmeasured groups in paleoclimatic reconstruction. Small non-equilibrium

offsets in the ^{13}C - ^{18}O compositions in echinoids may be corrected. Nautili however display inter-individual variation in ^{13}C - ^{18}O such that correction for non-equilibrium effects may be not be possible, precluding the use of the clumped isotopic composition of groups such as the ammonites in paleoclimatic reconstruction.

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Chapter 1: Introduction

1.1 GENERAL INTRODUCTION

The stable oxygen isotopic composition of calcifying marine organisms is widely used to reconstruct temperature records of ancient oceans (Emiliani, 1955; Zachos, 2001). The stable oxygen isotopic composition of some taxa faithfully reflect both the temperature and the $\delta^{18}\text{O}$ of the seawater in which the organism grew. Other taxa however show species-specific discrepancies known as “vital effects” (Epstein et al., 1951).

‘Vital effects’ (Urey, 1951), otherwise known as physiological effects (Epstein et al., 1951) or biological fractionations, refer to the offset between the isotopic composition of biological carbonates and that expected from equilibrium precipitation in seawater. They may be widely split into two groups; kinetic effects relating to varying kinetics of the uptake, transport of deposition stages of mineral formation (McCrea, 1950; McConnaughey, 1989) and metabolic or taxonomic effects which relate to the biological character of the organism and the way in which they facilitate carbonate formation including local influences on partitioning of dissolved inorganic carbonate in solution and/or incorporation of metabolic CO_2 (Weiner and Dove, 2003).

Vital effects pose a significant barrier to paleoenvironmental reconstructions by stable isotope geochemistry. However with the exception of a few significant studies (Erez 1978, McConnaughey 1989, Spero et al. 1991, Ziveri et al. 2003, Adkins 2003) the mechanisms of vital effects and how they relate to organism biology remain relatively uninvestigated. The primary method of circumventing the problem of the ‘vital effect’ is to study groups including extant species that demonstrate equilibrium deposition with seawater for a given isotopic system. This approach however assumes that the relationship between isotopic composition and a given environmental parameter has remained constant through time and that there is no impact of ecological factors or ontogenetic changes on isotopic composition.

Carbonate clumped isotope geochemistry is a powerful new paleotemperature proxy that relies on the thermodynamically based “clumping” of ^{13}C - ^{18}O bonds within the carbonate lattice (Ghosh et al., 2006; Eiler, 2007; Dennis et al., 2011). A distinct advantage of clumped isotope geochemistry over the traditional oxygen-isotope carbonate-water thermometry is

the ability to reconstruct carbonate formation temperature independent of the oxygen isotopic composition of water in which the carbonate formed (Eiler, 2007).

The first biogenic calibrations indicated that carbonate clumped isotope thermometry may be free of the species-specific “vital effects” that plague stable oxygen isotope paleothermometry (Ghosh et al., 2006; Tripathi et al., 2010; Thiagarajan et al., 2011). Groups such as coccoliths and deep-sea corals, which exhibit strong disequilibrium signatures in $\delta^{18}\text{O}$ composition, show conformable relationships between ^{13}C - ^{18}O clumping in biogenic and inorganic calcite (Thiagarajan et al., 2011, Katz et al. 2017).

More recently studies have highlighted subtle non-equilibrium effects in the clumped isotope composition of brachiopod molluscs (Henkes et al., 2013; Bajnai et al., 2018) corals (Saenger et al., 2012; Kimball et al., 2015; Spooner et al., 2016) and cephalopod molluscs (Dennis et al., 2013). Bajnai et al. (2018) recently highlighted the importance of kinetic controls on the clumped and $\delta^{18}\text{O}$ composition of modern brachiopods. Kinetic effects during hydration/dehydration of CO_2 are also postulated as a contributor to vital effects in surface corals (Saenger et al., 2012). However the relative contributions of kinetic and taxonomic metabolic effects controlling disequilibrium precipitation in other groups remain uncertain.

The comparison of the relative magnitude of disequilibrium in varying isotopic systems during carbonate formation may help in furthering our understanding of mechanisms that produce vital effects. For example correlated plots of ^{13}C - ^{18}O bond abundance, $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ in biological carbonate may be compared with predicted correlations based on modelled and/or experimentally determined kinetics relating pH effects and salinity (Tripathi et al. 2015), CO_2 diffusion (Thiagarajan et al. 2011) or CO_2 hydration and hydroxylation reactions (Guo, 2008) (Figure 1.1).

A more mechanistic understanding of vital effects increases proxy reliability especially when there is a need to differentiate between equilibrium precipitation and small non-equilibrium effects. Furthermore it enables workers to be more predictive in determining which isotopic systems will be affected by disequilibrium precipitation. This brings the community closer to deconvolving isotopic signals in biological carbonate enabling climatic reconstructions from primary climatic signals whilst accounting for secondary biological overprints.

Finally carbonate clumped isotope geochemistry, in comparison with stable oxygen isotope thermometry, is still undergoing rapid expansion and methodological refinement. This study enables us to test the clumped isotope composition of previously unmeasured groups such as the echinoids and re-visit initial measurements of cephalopods produced prior to recent advances in laboratory methods.

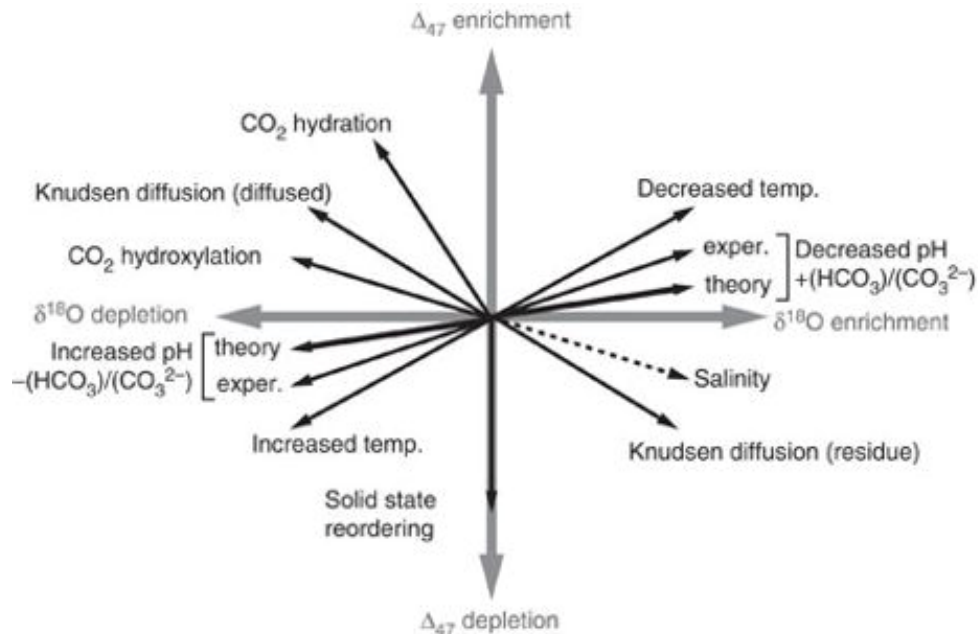


Figure 1.1 Vector trajectories in $\delta^{18}\text{O}$ and Δ_{47} for effects of different processes on stable isotope signatures such as pH effects and salinity (Tripathi et al., 2015), CO_2 diffusion (Thiagarajan et al., 2011), CO_2 hydration and hydroxylation reactions (Guo, 2008) from (Lloyd et al., 2016).

1.2 PROJECT AIMS

The main aim of this study is to test how robust the clumped isotope thermometer is to biologically mediated non-equilibrium precipitation. This will contribute answering the general question as to whether non-equilibrium signatures in Δ_{47} are an exception or the norm in biological carbonates. We seek to assess the clumped isotopic composition of groups such as the echinoids and modern cephalopods whose clumped isotope composition is previously uninvestigated or underreported in order to determine if they deposit their shells in at ^{13}C - ^{18}O clumped isotope equilibrium.

1.3 DETAILED OBJECTIVES AND APPROACH

1) Test the effects of contaminant molecules on the relative abundance of the mass 47 isotopologue

- Determine correlation between Δ_{47} and established metrics of contamination Δ_{48} offset and 49 parameter. Use these correlations to define threshold values below which samples can be reliably considered “uncontaminated”.
- Experiment with methods to improve CO₂ purification such as lowering the temperature of the Porapak Q U-trap
- Test sample pre-treatment methods such as low temperature oxygen plasma ashing to circumvent the need for sample pre-treatment using wet chemistry

2) Measure the clumped isotopic composition of echinoid samples with different growth temperatures

- Test to determine if there are inter-skeletal differences in the clumped isotope composition of echinoids reflecting differences reported in $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$
- Calibrate the Δ_{47} temperature relationship for the echinoids
- Compare the relative magnitude of any observed disequilibrium effects in Δ_{47} with disequilibrium effects in $\delta^{18}\text{O}$

3) Measure the Δ_{47} composition of cephalopods building on the work of Dennis et al. (2009)

- Investigate the Δ_{47} temperature relationship in cephalopods by studying the Δ_{47} values of Nautili and Sepia grown at known temperature
- Measure Δ_{47} and $\delta^{18}\text{O}$ values of Nautili septa from juvenile septa to last formed septa
- Determine if Δ_{47} and $\delta^{18}\text{O}$ in Nautili and Sepia are precipitated in equilibrium with seawater

1.4 THESIS OUTLINE

This thesis consists of six chapters. Chapter 3 to 5 are presented as papers, chapter 3 illustrating improvements in methodological aspects for clumped isotope analysis, chapters 4 and 5 presenting vital effects in two distinct groups, the echinoids and cephalopods.

Chapter 1: “General Introduction”. This chapter; presenting introduction and background, project aims, detailed objectives and approach

Chapter 2: “Methodology” This chapter provides background to the clumped isotope measurement including inorganic calibration of the clumped isotope thermometer, instrumental set up and methodology in the Qatar Stable Isotope Laboratory at Imperial College London. In this chapter I also present the statistical methods used in this study.

Chapter 3: “Reducing Contamination Parameters for Clumped Isotope Analysis: The effect of lowering Porapak Q trap temperature to below

-50° C”. When comparing measuring the clumped isotopic composition of biogenic and inorganic carbonate it is important that CO₂ measured is free of contaminant molecules that produce isobaric interference within the mass range of the mass 47 isotopologue of CO₂. This chapter thus focuses on identification of sample contamination and contaminant reduction during the purification of analyte CO₂.

This chapter was published in 2017 in *Rapid Communications in Mass Spectrometry*:

Davies A. J. and John C. M. (2017) Reducing contamination parameters for clumped isotope analysis: The effect of lowering Porapak™ Q trap temperature to below –50°C. *Rapid Commun. Mass Spectrom.* **31**, 1313–1323.

Chapter 4: The clumped (¹³C-¹⁸O) isotope composition of echinoid calcite: further evidence for “vital effects” in the clumped isotope proxy. This chapter focuses on determination of vital effects in the clumped isotopic composition of echinoids. This includes the first comprehensive report of echinoid clumped isotope composition.

This chapter was published online in *Geochimica et Cosmochimica Acta* (27th July 2018):

Davies, A.J. & John, C.M. 2018. The clumped (¹³C-¹⁸O) isotope composition of echinoid calcite: further evidence for “vital effects”; in the clumped isotope proxy. *Geochimica et Cosmochimica Acta*, 1–41, doi: 10.1016/j.gca.2018.07.038.

Chapter 5: The clumped (¹³C-¹⁸O) isotope composition of modern cephalopod calcite: non-equilibrium precipitation in the clumped isotope composition of biogenic calcite.

This chapter comprises Δ_{47} , $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ of modern cephalopods. Here we expand current understanding of the nature of non-equilibrium Δ_{47} signals in cephalopod aragonite.

The study is to be submitted to *Chemical Geology*:

Davies A.J., Davis S., & John C.M The clumped (¹³C-¹⁸O) isotope composition of modern cephalopod calcite: non-equilibrium precipitation in the clumped isotope composition of biogenic calcite.

Chapter 6: Conclusions and Further Work This chapter ties studies in chapters 3 4 and 5 together to discuss positive evidence for vital effects in the clumped isotope thermometer of groups measured and contrast biomineralization strategy of both groups with vital effect mechanisms.

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Chapter 2: Background and Methodology

1) BACKGROUND

1.1 The theory of multiply substituted isotopologues

Isotopologues are molecules, organic moieties or mineral structural units with the same constituent atoms but varying isotopic identity for example H_2 and HD or $^{12}\text{C}^{16}\text{O}_3^{2-}$ and $^{13}\text{C}^{16}\text{O}_3^{2-}$. Isotopologues containing two or more heavy isotopes i.e. D_2 and $^{13}\text{C}^{18}\text{O}^{16}\text{O}$ are referred to as multiply substituted isotopologues. Clumped isotope geochemistry assesses the state of ordering and abundance of rare “heavy” isotopes, specifically the degree to which heavy isotopes bond together or within close proximity of one another in natural materials. Clumped isotope geochemistry thus comprises the measurement of multiply substituted isotopologues in molecules and minerals (Eiler, 2007).

The theoretical principles on which stable isotope geochemistry is based were first established in the 1920's - 1940's. The most important discovery during this period was that isotopic substitution of a heavy for light isotope, i.e. substitution of H-D for H-H, in a chemical bond reduces the vibration frequency of that bond therefore its zero point energy (Urey, 1947; Bigeleisen and Mayer, 1947) (Figure 2.1). Indeed the difference in vapor pressures between hydrogen molecules, H_2 and HD , was measured as early as 1931 (Urey et al., 1931). The “rule of geometric mean” implies that the decrease in bond energy associated with heavy-heavy substitutions (i.e. substitution from H-H to D-D) is twice that of the heavy substitution of only one atom (eg H-D) (Bigeleisen, 1955). This is however only an approximation, as decreases in vibrational energy associated with double heavy-isotope substitution frequently slightly exceed twice that of a single heavy-isotope substitution (Urey 1941, Bigeleisen 1955), resulting in a thermodynamic driving force that promotes the “clumping” of heavy isotopes at the expense of singly-substituted isotopologues.

This may be further examined by considering the isotopic disproportionation reaction:



Where the equilibrium constant $K_1 = ([\text{H}_2] \cdot [\text{D}_2]) / [\text{HD}]^2 = ([\text{H}]_2 \cdot [\text{D}]_2) / (2 \cdot [\text{H}] \cdot [\text{D}])^2 = 0.25$. However as D_2 is slightly more stable than predicted based on the rule of the mean, the forward reaction in this homogenous isotope exchange equilibrium is favored thus raising K_1 .

This is balanced by the increase in entropy produced by isotopic distribution. A tendency of increase in entropy (random distribution of rare isotopes) with increased temperature in combination with generally decreased enthalpies of isotopic exchange results in increased in K_1 with lower temperature (Figure 2.2) thus the promotion of higher proportional abundances of multiply substituted isotopologues (Eiler 2007).

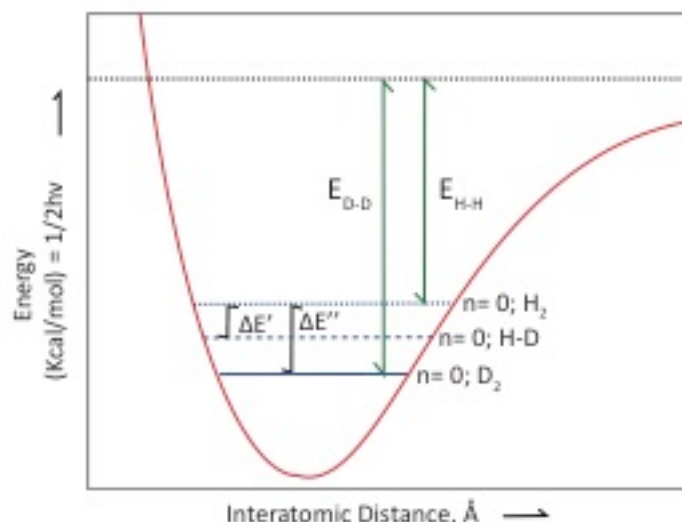


Figure 2.1 Energy of a hydrogen bond (y axis) against the bond distance at minimum energy-bond distance (x axis). Red line shows the Morse potential for H_2 . Horizontal represent the quantum states of energy, where $n=0$ is the zero point energy (ZPE) $\Delta E'$ and $\Delta E''$ give differences in energy between H-D and H_2 , D_2 and H_2 respectively (Eiler, 2007).

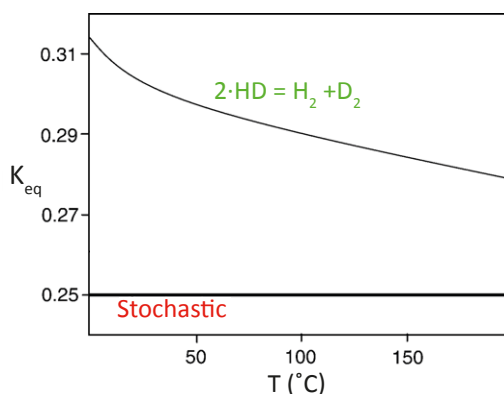


Figure 2.2 Temperature dependence of the equilibrium constants for the homogeneous isotope exchange reaction (Equation 1). The heavy horizontal line indicates the value of the equilibrium constant that corresponds to a stochastic distribution (0.25). Note that the gradual approach of K_{eq} toward the stochastic distribution with increasing temperature. Equilibrium curves are taken from (Richet et al., 1977) (Eiler, 2007).

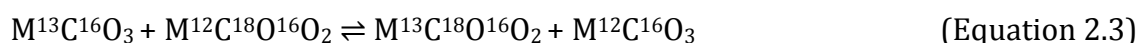
Clumped isotope analyses are reported in terms of Δ_i which denotes the excess or deficit of specific isotopologue i relative to the stochastic distribution (Eiler, 2007).

$$\Delta_i = \left(\frac{R_i}{R_{i^*}} - 1 \right) \cdot 1000 \quad (\text{Equation 2.2})$$

The stochastic distribution, in which all isotopes are equally distributed amongst isotopologues, thus defines a reference frame against which deviations in heavy-heavy bonded or “clumped” isotope abundance can be measured.

1.2 Carbonate Clumped Isotope Thermometry

Carbonate clumped isotope thermometry specifically examines preference of heavy isotopes ^{13}C and ^{18}O to bond or “clump” together within the carbonate crystal lattice. This is based on the thermodynamically controlled homogenous isotope exchange equilibrium:



(where M is a metal such as Ca) favouring the formation of $\text{M}^{13}\text{C}^{18}\text{O}^{16}\text{O}_2$ with decreasing temperature (Schauble et al., 2006). As ^{13}C - ^{18}O isotopic clumping ensues via the above self-reaction of isotope exchange, a reaction occurs rapidly at near neutral pH, the clumped isotopic composition of the carbonate is independent of the oxygen isotopic composition parent fluid (Eiler, 2007). The $^{13}\text{C}^{18}\text{O}^{16}\text{O}$ isotopologue constitutes approximately 97% of the abundance of CO_2 measured on mass 47 (Eiler, 2007).

$^{12}\text{C}^{16}\text{O}_2$	98.40%
$^{13}\text{C}^{16}\text{O}_2$	1.11%
$^{12}\text{C}^{17}\text{O}^{16}\text{O}$	748 ppm
$^{12}\text{C}^{18}\text{O}^{16}\text{O}$	0.40%
$^{13}\text{C}^{17}\text{O}^{16}\text{O}$	8.4 ppm
$^{12}\text{C}^{17}\text{O}_2$	0.142 ppm
$^{13}\text{C}^{18}\text{O}^{16}\text{O}$	44.4 ppm
$^{12}\text{C}^{17}\text{O}^{18}\text{O}$	1.50 ppm
$^{13}\text{C}^{17}\text{O}_2$	1.60 ppb
$^{12}\text{C}^{18}\text{O}_2$	3.96 ppm
$^{13}\text{C}^{17}\text{O}^{18}\text{O}$	16.8 ppb
$^{13}\text{C}^{18}\text{O}_2$	44.5 ppb

Figure 2.3 Isotopologue abundances of CO_2 given a stochastic distribution (Eiler, 2007)

As quantification of ^{13}C - ^{18}O ordering currently requires a gas analyte, the carbonate is acid digested to produce CO_2 in which the abundance of the $^{13}\text{C}^{18}\text{O}^{16}\text{O}$ isotopologue is proportional to the extent of ^{13}C - ^{18}O ordering in the carbonate phase (Ghosh et al., 2006). The relative abundance of ^{13}C - ^{18}O clumping in carbonates is defined by the metric Δ_{47} . The excess of $^{13}\text{C}^{18}\text{O}^{16}\text{O}$ in analyte CO_2 relative to a stochastic distribution is expressed using the metric Δ_{47} defined as:

$$\Delta_{47} = \left(\frac{R^{47}}{R^{47*}} - 1 \right) - \left(\frac{R^{46}}{R^{46*}} - 1 \right) - \left(\frac{R^{45}}{R^{45*}} - 1 \right) \times 1000$$

(Equation 2.4)

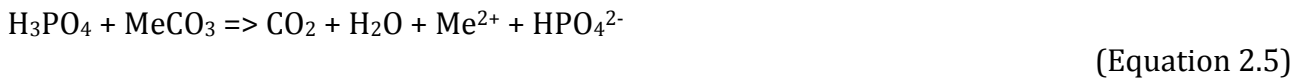
(Eiler and Schauble, 2004) where $R_i = m_i/m_{44}$ and R^* gives the abundance of each isotopologue of a gas with the same bulk isotopic composition as R but with a stochastic distribution (Affek and Eiler, 2006).

2.METHODOLOGY

This chapter details the general methodology of the clumped isotope measurement at Imperial College London (ICL). The way in which the clumped isotope measurement was made at ICL has evolved in the period 2014-2018 thus chapters 3-5 include brief explanations of the exact methodology used in each individual study.

2.1 CO₂ extraction and purification

In this study carbon dioxide extraction and purification was achieved off-line using two cryogenic manual purification lines in the Qatar Stable Isotope Laboratory at ICL. Samples weighing 5-10 mg were reacted in 105% anhydrous orthophosphoric acid under vacuum to produce analyte CO₂ (McCrea, 1950; Swart et al., 1991). The reaction occurs as follows:



where Me is a metal cation (e.g. Ca²⁺). This reaction was allowed to proceed for 10 minutes for calcite samples and 20 minutes for dolomite samples. Throughout this period product CO₂ was cryogenically trapped in a glass spiral trap immersed in liquid nitrogen (LN₂).

Product CO₂ was then purified to remove contaminant gasses, produced on acid digestion of the carbonate, such as short chain halocarbons, hydrocarbons, sulfide gasses and water vapor.

Manual CO₂ purification

This was achieved by passing the CO₂ through a silver trap, a static Porapak Q (PPQ) glass U-trap, and a series of water traps (Figure 2.4). Porapak QTM is a divinyl benzene polymer produced by Sigma-Aldrich, designed for use in gas chromatography columns separating polar gasses such as SO₂ from non-polar CO₂. Following cryogenic trapping during acid reaction in trap 1 non-condensable gasses were pumped away. CO₂ was then released by

applying ethanol slush at -90°C to trap 1 and passed through a $\frac{1}{4}$ inch outer diameter glass sliver trap containing approximately 7 g of fine grade silver wool (89 scqc/g) manufactured by Elemental Microanalysis. Treatment of analyte CO_2 with elemental silver or Ag_3PO_4 to remove and sulfur compounds has been previously documented (Guo and Eiler, 2007; Eagle et al., 2010). Migration of CO_2 across the PPQ trap is driven by both temperature differential created by the pull of LN_2 applied to trap 3 and pressure differential from the high vacuum pump. The temperature of the PPQ trap was maintained at -35°C unless otherwise discussed. CO_2 transfer is maintained until pressure on gauge 1 reads 1.8×10^{-2} . Over the period 01/10/13 – 22/11/2017 transfer time across the PPQ trap took between 40 and 60 minutes. Transfer time following a change of the porapak Q within the U-trap on 20/11/2017 decreased to 20 minutes with no evidence of a reduction in cleaning efficiency. CO_2 was cryogenically trapped in trap 3 before again being released by application of ethanol slush at -90°C and frozen into the cold finger (trap 4) for transfer to the mass spectrometer.

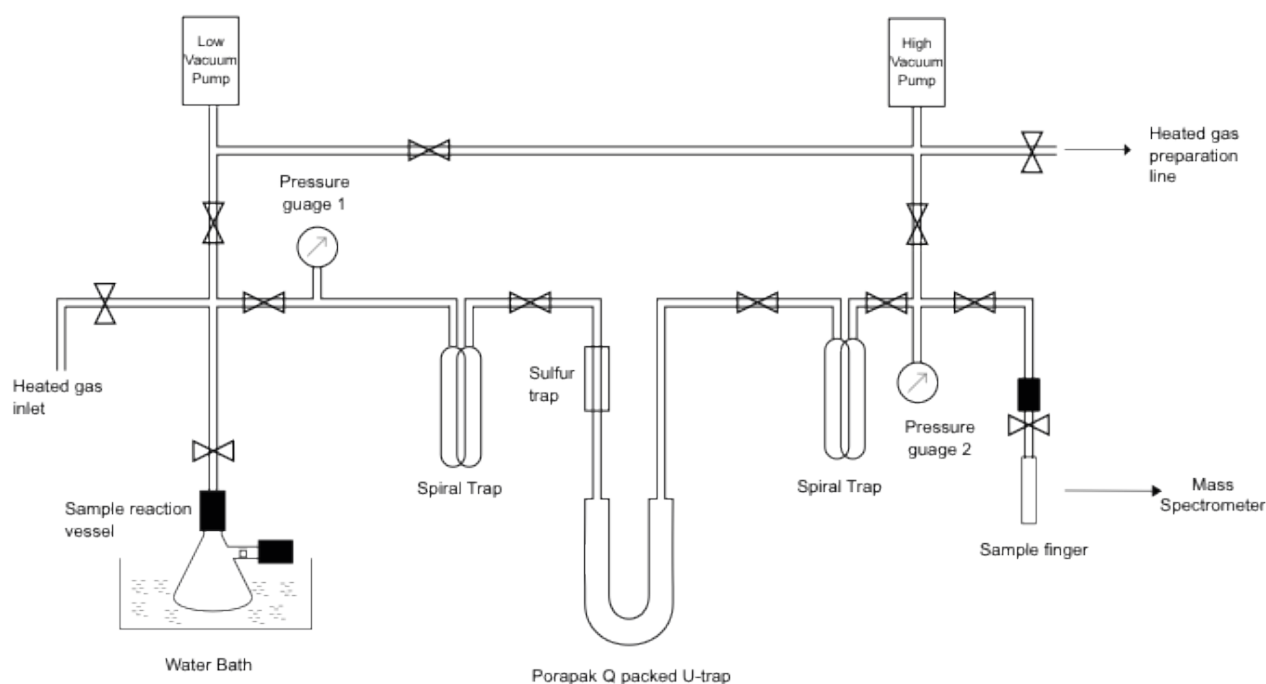


Figure 2.4 Illustration of acid digestion and manual cryogenic purification line. Spiral trap temperature is controlled by the application of liquid nitrogen or a liquid nitrogen and slush mixture at -90°C . Porapak Q U-trap is heated by surrounding copper wire coil across which a voltage is applied. Copper tape wrapping dipped in liquid nitrogen draws heat away an attached thermocouple providing temperature feedback enabling trap temperature to be maintained at any value between 220°C and liquid nitrogen temperature.

In between CO_2 purifications the PPQ trap was heated to 140°C under vacuum for at least 35 minutes to promote the desorption and removal of contaminant molecules. A hot air gun was used to heat water traps and metal tubing to over 150°C promoting the evaporation and

removal of residual water. Standard stable pressure on the line between purifications was $<3 \times 10^{-3}$.

Automated batch acid digestion and CO₂ purification (IBEX)

In contrast with manual sample preparation samples measured on the IBEX (Figure 2.5) were batch digested in 8 ml of 105% orthophosphoric acid. Samples were delivered to the acid reaction vessel by a rotating auto sampler and the evolved CO₂ was frozen down continuously during the 10 minute acid digestion period. Analyte CO₂ was released by heating the first water trap to -80°C and transferred across a 1 m long 2.5 mm inner diameter GC column held at -35°C, using helium as carrier gas with a flow rate of 35 ml per minute and pressure of 2 psi. The CO₂ was then frozen in a second trap at -192°C, and cryogenically transferred into a micro-volume prior to its introduction into the bellows of mass spectrometer Pinta. Total time taken for sample preparation on the IBEX was approximately 40 minutes.

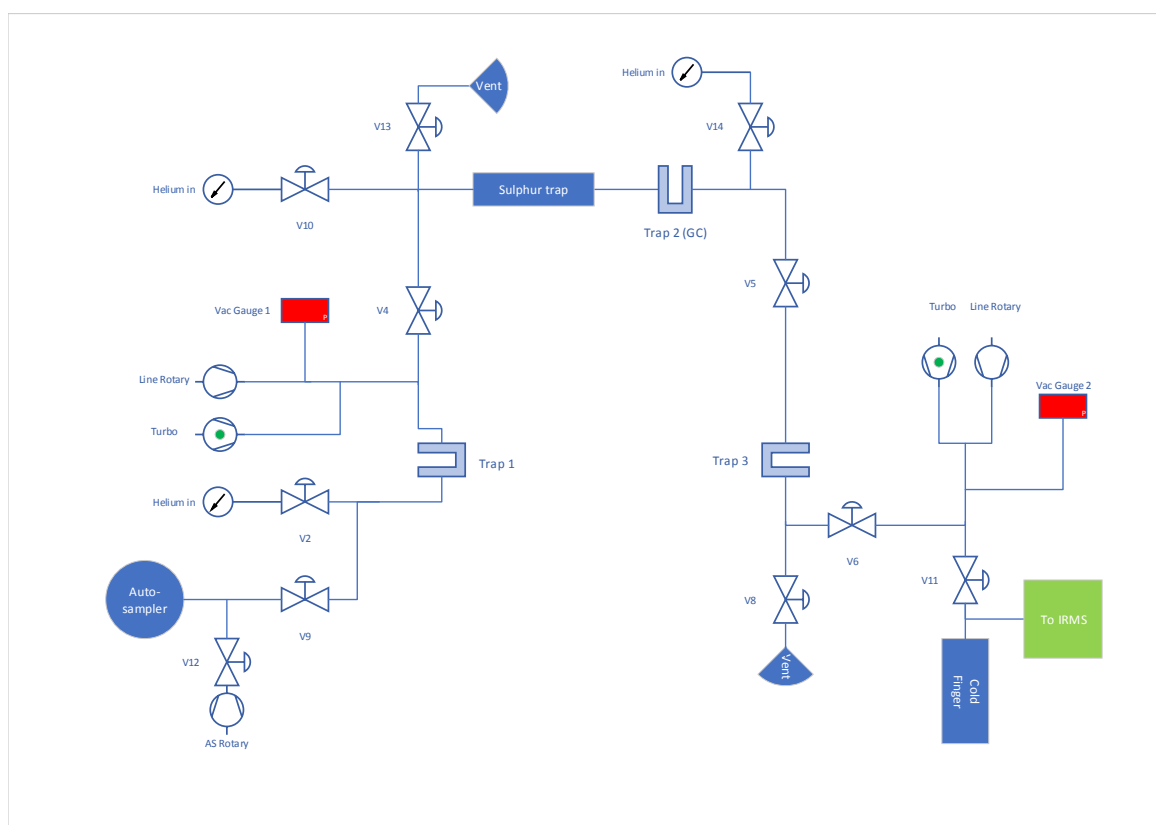


Figure 2.5 Illustration of the IBEX automatic clumped isotope measurement device. Samples are batch acid reacted and purified through a GC column by active helium flow.

2.2 Mass Spectrometry

Sample isotopic compositions were measured, in the Qatar Stable Isotope Laboratory at Imperial College London, using three Thermo 253 gas source IRMS systems denoted 'Nina', 'Pinta' and 'Santa Maria' (Thermo Instruments Bremen, Germany) (Figure 2.6). These mass spectrometers are equipped with a dual inlet system that allows rapid repeated inter-comparison between sample gas and a reference gas of known isotopic compositions (Huntington et al., 2009). Each mass spectrometer has collection systems consisting of standard cups registered through m/z 44-46 and three additional cups registered for m/z 47, 48 and 49 as detailed by (Eiler and Schauble, 2004) (Figure 2.7).

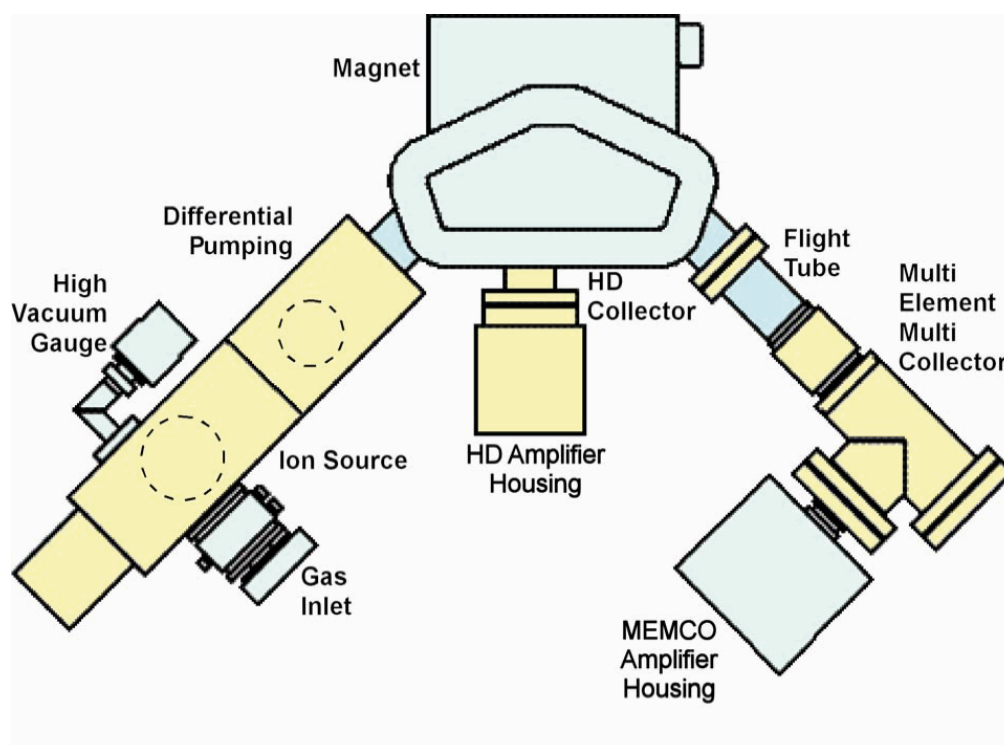


Figure 2.6 Diagram of a gas source Thermo MAT 253 mass spectrometer (ThermoFinnigan, 2002)

Gas Configuration Editor



Add



Delete

Configurations

Name	Cup1	Cup2	Cup3	Cup4	Cup5	Cup6	Cup7	Cup8	Calibration	Ratio Groups	Magnet	PC-Offset
N2		28	29	30					Current [Default]	N2	3822	0
CO		28	29	30					Current [Default]	CO	3822	0
Clumped		44	45	46		47	48	49	Current [Default]	CO2	5644	0
CO2		44	45	46		47	48	49	Current [sd]	CO2	5717	0
Mass_47_On		44	45	46		47	48	49	Current [simon ibex]	CO2	5823	0

Mass Info

Number of required cups : - Masses required : -

Save & Close

Cancel

Figure 2.7 Cup configurations for "Pinta" (Santa Maria has an identical configuration)

The majority of measurements for this thesis were made on Pinta, in use since 2012 with later measurements from November 2017 onwards acquired on Santa Maria. Both machines are fit with deactivated fused silica capillaries (SGE Australia) with 1 m length 110 μm internal diameter and 320 μm outer diameter.

Oztech was initially used as a reference/working gas owing to its proximity in bulk isotopic composition to samples gases measured. In November 2017 Oztech no longer became available, thus the reference gas was switched to a well-characterized high purity (HP) carbon dioxide. The $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ of the Oztech and HP reference gas values used are given in chapters 3-5 of this study.

Measurements consisted of 8 acquisitions each with 7 cycles with 26 s integration time. On introduction of the sample to the mass spectrometer, bellow pressure was adjusted to approximately 45 mbar to generate a m/z 44 ion beam signal of roughly 15 000 mv that was maintained throughout the analysis by constant adjusting. Before each acquisition peak-centering and pressure adjustments were automatically carried out. A typical acquisition time was 16 minutes corresponding to a total analysis time of around 2 hours 10 minutes.

Internal precision measures the reliability of the mass spectrometer in isolation based on repeated analysis of the same aliquot of gas. Internal precision of the mass spectrometer depends on random (shot-noise) errors and uncharacterized mass spectrometric fractionations such as isotopic exchange of CO_2 molecules within the source. The shot noise limit to internal precision using 15 kv signal and 1664 second integration is calculated as 0.009‰ (3sf) (Merritt and Hayes, 1994). The average standard error of 20-heated gas Δ_{47} measured on mass spectrometer ‘Pinta’ over the period 26.10.13-06.03.14 is 0.0109‰ $\pm$ 0.0008‰ supporting the suggesting that internal precision is primarily controlled by counting statistics (Huntington et al. 2009).

2.3 Data Processing and Correction

2.3.1 Calculation of $\delta^{18}\text{O}$, $\delta^{13}\text{C}$, raw Δ_{47} and Δ_{48} of sample gas

All calculations for this work were made using the freely available software Easotope, developed within the carbonate research group (John and Bowen, 2016). First we calculate the relative abundance ratios R_{13} , R_{17} and R_{18} of the working gas (WG) based on known

working gas $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ where $R_{13} = {}^{13}\text{C}/{}^{12}\text{C}$, $R_{17} = {}^{17}\text{O}/{}^{18}\text{O}$ and $R_{18} = {}^{18}\text{O}/{}^{16}\text{O}$ of the gas and an international reference material, in this case VSMOW and VPDB.

$$R_{13WG} = R_{13VPDB} \times \left(1 + \frac{\delta^{13}C_{WG}}{1000}\right) \quad (\text{Equation 2.6})$$

$$R_{18WG} = R_{18VSMOW} \times \left(1 + \frac{\delta^{18}O_{WG}}{1000}\right) \quad (\text{Equation 2.7})$$

$$R_{17WG} = R_{17VSMOW} \times \left(1 + \frac{R_{18WG}}{R_{18VSMOW}}\right)^{\lambda} \quad (\text{Equation 2.8})$$

Before 2016 the Santrock parameters for R_{13VPDB} , R_{17REF} , R_{18REF} and λ , the terrestrial mass dependent fractionation factor between ${}^{17}\text{O}$ and ${}^{18}\text{O}$, (Gonfiantini et al., 1995) were used in calculation. However, following findings (Daeron et al., 2015; Schauer et al., 2016) we switched to use of the Brandt parameters (Brand et al., 2010) to reduce systematic errors in Δ_{47} controlled by the bulk isotopic composition of samples and standards.

	Santrock Parameters (Gonfiantini et al., 1995)	Brandt Parameters (Brand et al., 2010)
R_{13VPDB}	0.0112372	0.01118
$R_{17VSMOW}$	0.0003799	0.00038475
$R_{17VPDB-CO_2}$		0.00039310
$R_{18VSMOW}$	0.0020052	0.0020052
$R_{18VPDB-CO_2}$		0.00208839
λ	0.5164	0.528

Abundances of ${}^{12}\text{C}$, ${}^{13}\text{C}$, ${}^{16}\text{O}$, ${}^{17}\text{O}$ and ${}^{18}\text{O}$ were computed for the working gas prior to calculating the isotopologue composition of the working gas, mass 44-49 ($R_{44-R_{49}WG}$), assuming a stochastic distribution. Isotopologue abundances of the working gas were then binned by mass and normalised by the most abundant mass 44 isotopologue. For further details of these calculations see John and Bowen (2016).

We could then compute the abundance ratios by mass of the sample gas ($R_{44-R_{49}sample}$)

$$R_{mi\ sample} = R_{mi\ WG} \frac{V_{mi\ sample}}{V_{mi\ sample}} \times \frac{R_{44\ WG}}{R_{mi\ WG}} \quad (\text{Equation 2.9})$$

where R_{mi} is the mass abundance ratio for example R_{44} and V_{mi} is measured voltage in millivolts. The relative abundance ratios R_{13} , R_{17} and R_{18} of the sample gas were then computed by numerically solving the equation

$$-3 K^2 (R^{18})^k + 2 K R^{45} (R^{18})^k + 2 R^{18} - R^{46} = 0 \quad (\text{Equation 2.10})$$

using a second-order Taylor polynomial where R^{18}_{sample} is $(^{18}\text{O}/^{16}\text{O})$ R^{45} and R^{46} are the sample gas ratios (45/44) and (46/44) and $K = R^{17}_{VSMOW} \times (R^{18}_{VSMOW})^\lambda$ (Santrock et al. 1985, Daeron et al. 2016). Following on from this we calculated $R^{17}_{sample} = K \times (R^{18}_{sample})^\lambda$ and $R^{13}_{sample} = R^{45} - 2R^{17}$.

These values may then be used to calculate the $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ values of the sample gas.

$$\delta^{18}\text{O}_{VSMOW\ sample} = \frac{R_{18\ sample} - R_{18VSMOW}}{R_{18VSMOW}} \times 1000 \quad (\text{Equation 2.11})$$

$$\delta^{13}\text{C}_{VPDB\ sample} = \frac{R_{13\ sample} - R_{13VPDB}}{R_{13VPDB}} \times 1000 \quad (\text{Equation 2.12})$$

Calculated values for R^{13} , R^{17} and R^{18} were then used to mathematically ‘scramble’ the sample gas to produce a gas of the same bulk isotope composition but with a stochastic distribution giving stochastic isotopologue abundances on masses 45-49, defined as R^{45*} , R^{46*} , R^{47*} , R^{48*} and R^{49*} Affeck and Eiler (2006).

$$R^{45*} = R^{13} + 2R^{17} \quad (\text{Equations 2.13})$$

$$R^{46*} = 2R^{18} + 2R^{13} R^{17} + (R^{17})^2 \quad (\text{Equations 2.14})$$

$$R^{47*} = 2R^{13} \times R^{18} \times 2R^{17} \times R^{18} + R^{13} \times (R^{17})^2 \quad (\text{Equations 2.15})$$

Measured R^{44-49} may then be input alongside calculated stochastic $R^{44*-49*}$ values to define raw Δ_{47} .

$$\Delta_{47\ raw} [\text{‰ WG}] = \left[\left(\frac{R^{47}}{R^{47*}} - 1 \right) - \left(\frac{R^{46}}{R^{46*}} - 1 \right) \left(\frac{R^{45}}{R^{45*}} - 1 \right) \right] \times 1000 \quad (\text{Equation 2.16})$$

Raw Δ_{48} , used to detect mass 48 anomalies relative to the stochastic distribution, was calculated using the same principle.

$$\Delta_{48raw} [\text{‰ WG}] = \left[\left(\frac{R^{47}}{R^{47*}} - 1 \right) - 2 \left(\frac{R^{46}}{R^{46*}} - 1 \right) \right] \times 1000$$

(Equation 2.17)

2.3.2 Data Correction Procedures

Raw Δ_{47} was corrected in three steps. Firstly correction was carried out in account for non-linearity of the mass spectrometer then Δ_{47} values were translated into the absolute reference frame of (Dennis et al, 2011) and finally correction was applied for acid digestion temperature (Figure 2.8). Corrections were applied to a specific measurement period or ‘correction interval’. This is a period of time where conditions in the mass spectrometer are assumed to have remained relatively constant (Huntington et al, 2009, John and Bowen 2016). All heated gasses, equilibrated gasses and carbonate standards within a given correction interval were used to correct sample Δ_{47} .

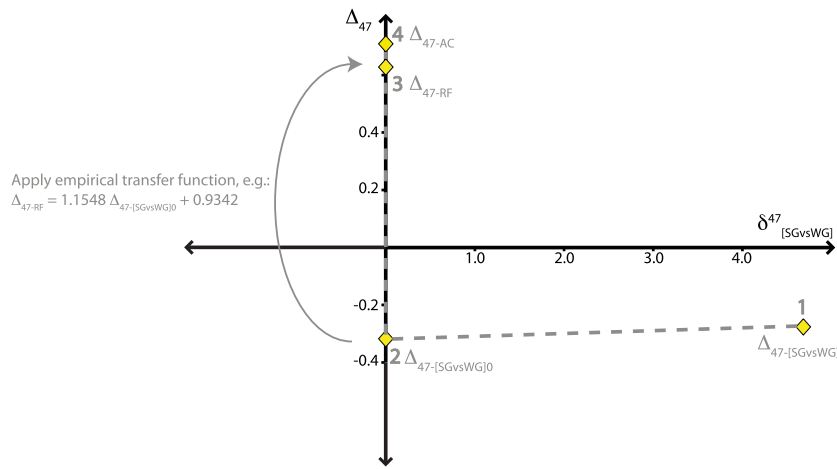


Figure 2.8 Schematic illustrating correction for raw Δ_{47} values. 1-2 shows the heated gas correction for non-linearity, 2-3 application of the empirical transfer function and 3-4 acid correction (Dennis et al., 2011).

2.3.3 Mass spectrometric non-linearity correction

Mass spectrometry involves a non-linearity between measured and real R^{47} values that may lead to significant errors in Δ_{47} especially if samples and standards differ significantly in isotopic composition (Ghosh et al, 2006; Huntington et al, 2010). The degree of non-linearity is variable depending on the mass spectrometer, time period of measurement and factors such as specific source conditions including cleanliness, filament and tuning (Bernasconi et al.

(2013). Non-linearity was corrected for by either a heated gas correction (Huntington et al. 2019) or pressure baseline correction (Bo He et al., 2002; Bernasconi et al., 2013)

Heated Gas correction

Heated gas correction was achieved by measuring the Δ_{47} gasses of varying bulk isotopic compositions (δ_{47}) prepared at 1000°C. Aliquots of carbon dioxide of δ_{47} between approximately -47 and 10 were prepared in quartz break seals and equilibrated to a near stochastic distribution by heating to 1000°C for >2 h followed by quenching at room temperature following the procedures of Huntington et al. (2009). Heated gases have a calculated Δ_{47} value of 0.027‰ (Wang et al. 2004) and are hereafter referred to as 'HG'.

Theoretically HG's prepared at 1000°C should have the same Δ_{47} . However mass spectrometric non-linearity results in increasing Δ_{47} at heavier δ_{47} (Figure 2.9). The heated gas line can be defined in the form $y = mx + c$ where; $x = \delta_{47}$, $y = \Delta_{47}$, m = gradient and c = y intercept value when $\delta_{47} = 0$. All Δ_{47} values are then projected to $\Delta_{47} = c$ thus correcting for mass spectrometric non-linearity.

$$\Delta_{47\text{sample}} = m(\delta_{47\text{sample}}) + c$$

(Equation 2.18)

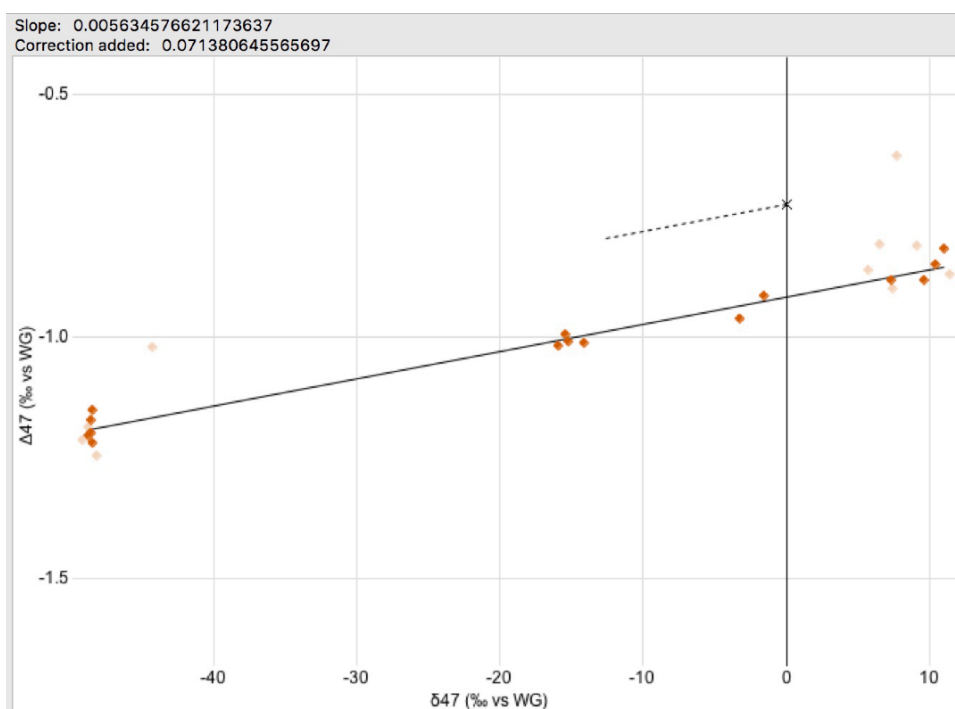


Figure 2.9 The heated gas slope ($[\delta_{47}\text{HG vs. } \delta_{47}\text{WG (‰)}]$) vs. ($[\Delta_{47}\text{HG vs. } \Delta_{47}\text{WG (‰)}]$) on mass spectrometer Pinta over the period 03/02/2017 to 21/08/2017.

Pressure Baseline (PBL) correction

In the Imperial College Qatar Stable Isotope Laboratory we have, since 2014, been experimenting with pressure baseline corrections as a way to better correct Δ_{47} data for mass

spectrometric non-linearity. Bo He et al. (2012) first noted how the drift in mass spectrometric non-linearity resulted from changing baseline signals on collectors m/z 47-49. We originally followed a slightly modified procedure to that reported by (He et al., 2012) using two 'off peak' pre-measurement cycles one 'off peak' post measurement cycle (John and Bowen, 2016) before switching to the approach of Bernasconi et al. (2013).

Following this approach we used a set of external "peak shape" scans, run daily, achieved by varying acceleration at partial pressures equating to mass intensities of between 12 kv and 17 kv on the 44 beam in approximately 1 kv steps (Figure 2.10) to correct for mass spectrometric non-linearity. Scans were saved and loaded into Easotope where the acceleration range of the background "pick" could be selected. The range where maximum peak height was selected was also manually selected in each scan, allowing for accurate selection in the case of lateral peak movement. Peak height "intensity of m/z 44-49 (mV)" was then plotted against minimum intensity in the selected background region for each mass "min of m/z 44-49 intensity (mV)" and a quadratic regression was fitted for the relationship on each mass 44-48 (Figure 2.11). Easotope then corrects for sample background using the regression fit of the nearest activated scan based on sample intensity (peak height) m/z 44-49 (mV).

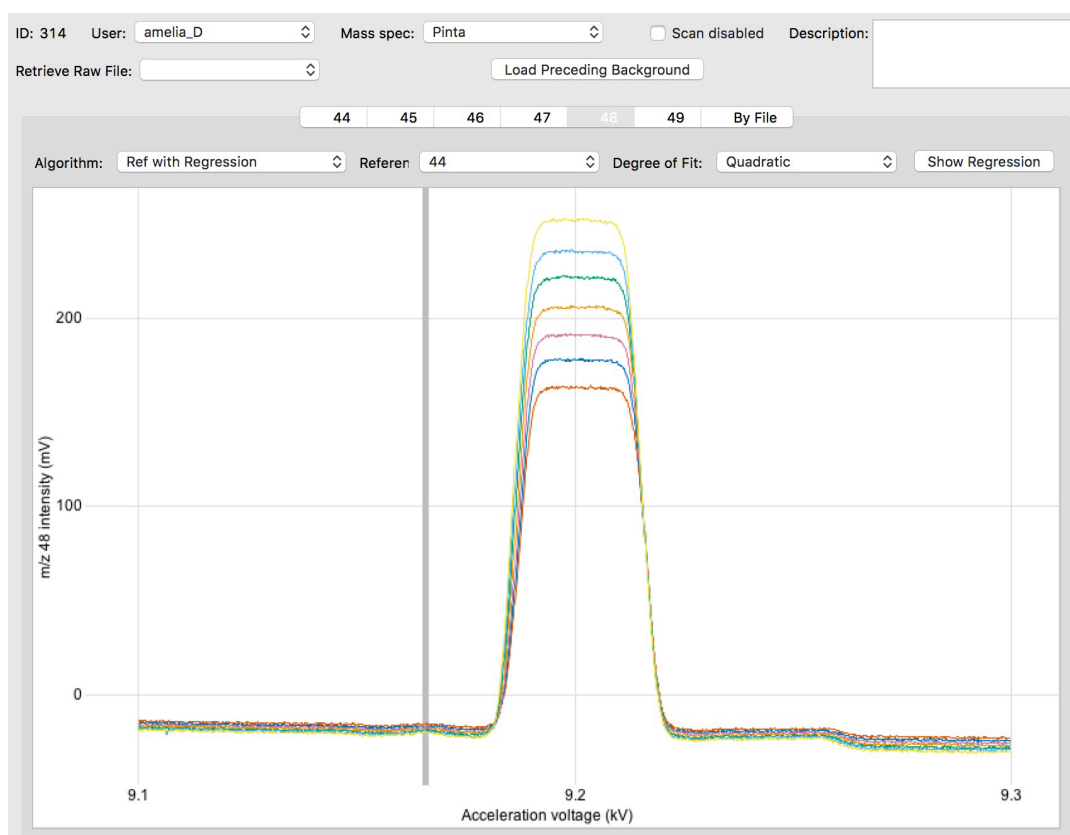


Figure 2.10 "Peak shape" scans at on mass spectrometer Pinta at 12 kv and 17 kv mass intensities on the 44 beam, showing mass 48 intensities across acceleration volatges 9.1 to 9.3

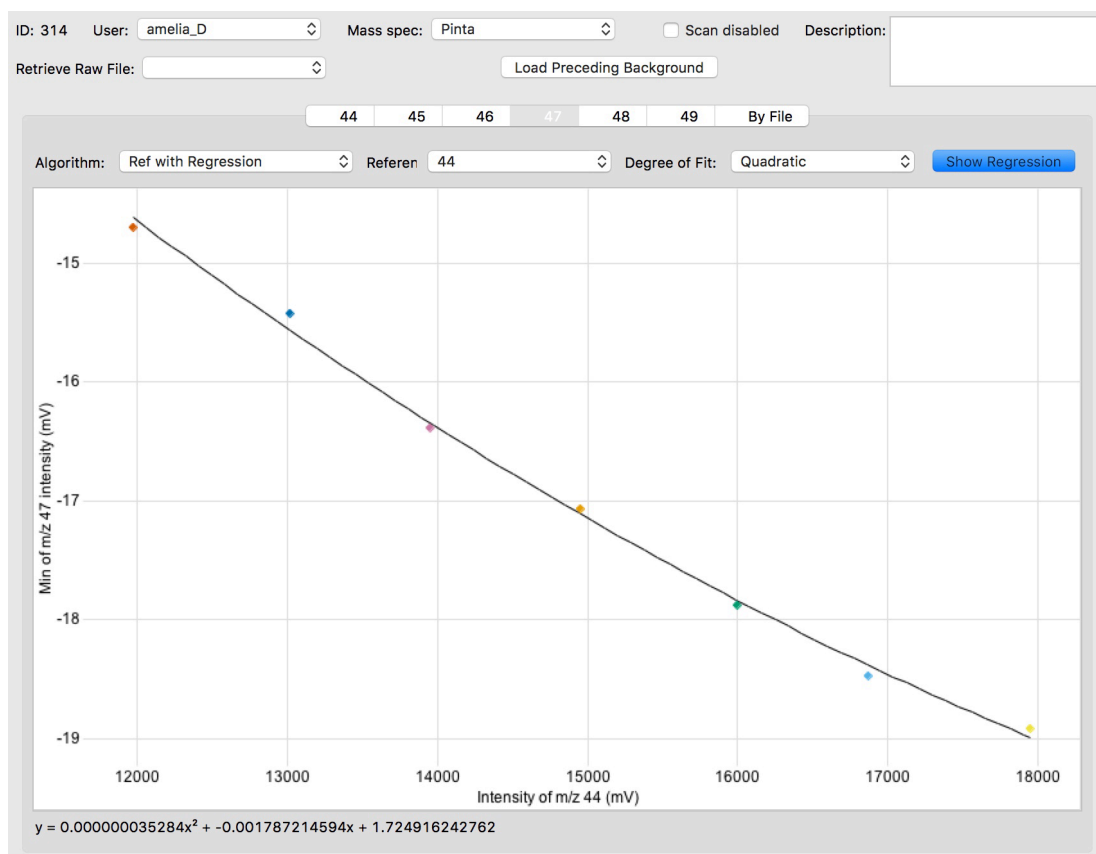


Figure 2.11 Regression fit of background on mass 47 (y axis) against intensity on m/z 44.

From 2017/02/03 onwards we provide both the HG corrected and PBL corrected values of samples measured on “Pinta” and “Santa Maria”. HG corrected sample values are given in chapter 4, however in chapter 5 we present the PBL corrected values. The move to PBL results from improved standard deviation on measured standards (Figure 2.12) and improvements in measuring smaller samples where partial pressure of the gas depletes significantly over the course of 8 acquisitions. This is a further step in the move to a carbonate only reference frame as intended at Imperial College Stable Isotope Laboratory.

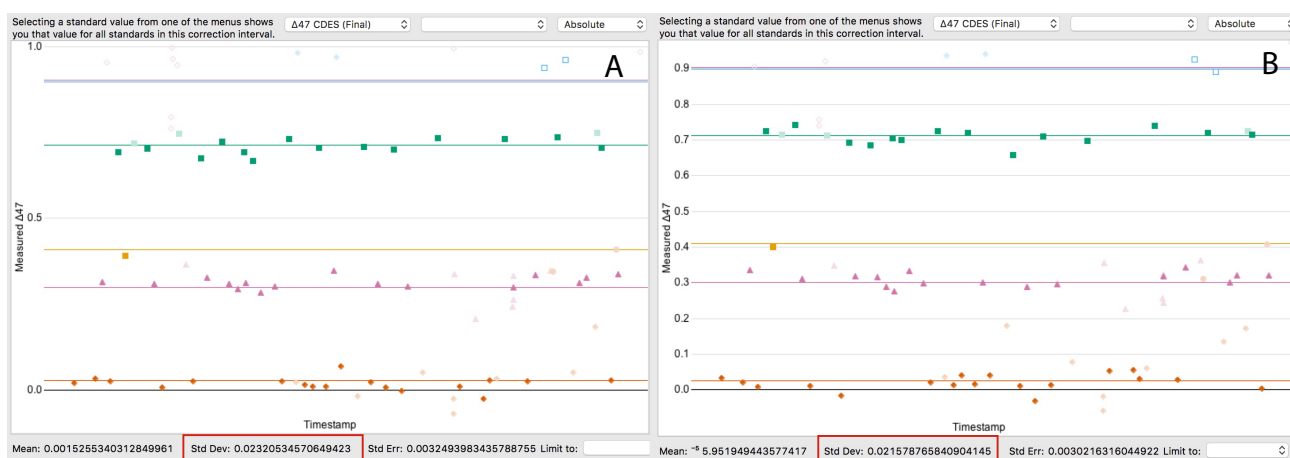


Figure 2.12 Comparison of final calculated Δ_{47} CDES of standards measured on Santa Maria over the period 27/03/2018-12/06/2018 corrected by heated gas correction, (A) and PBL correction (B) heated gas correction, in comparison with expected values.

2.3.4 Correction to the absolute reference frame of Dennis et al. (2011) (Δ_{47}^{CDES})

Following correction for mass-spectrometric non-linearity data is corrected to the absolute reference frame of Dennis et al. (2011) to allow for standardization of and inter-laboratory comparison between Δ_{47} values. During electron impact gas source mass spectrometry, fragmentation and recombination of CO_2^+ occurs in the source of the mass spectrometer leading to a redistribution of isotopes amongst isotopologues (Huntington et al., 2009). The absolute reference frame accounts for this effect, which is variable dependent on the source conditions and mass spectrometer.

Primary reference frame

Equilibrium of CO_2 in the gas phase can be experimentally established using commonly available materials whilst standardisation to the absolute reference frame is based on theoretical predictions of isotopic clumping of CO_2 at equilibrium (Wang et al., 2004). Equilibrated gasses were made by equilibrating and aliquot of CO_2 with 0.1 ml of water in a quartz break-seal, with $\frac{1}{4}$ inch diameter approximately 20 cm in length, at a given temperature in a water bath for several days. The δ_{47} of equilibrated CO_2 is dependent on $\delta^{18}\text{O}_{\text{water}}$ however Δ_{47} solely reflects equilibration temperature of equilibration. When using water with varying $\delta^{18}\text{O}_{\text{water}}$ the linear regression between δ_{47} and Δ_{47} of gasses equilibrated should have the same gradient as the heated gas line reflecting mass spectrometric non-linearity. At Imperial College London end members of tap water and evaporated water were used to make equilibrated gases, switching solely to tap water from 2016 onwards, to minimise Δ_{47} effects introduced by anomalies in ^{17}O abundance (Daëron et al., 2011).

The linear regression between $\Delta_{47}^{\text{EG(WG)}}$ and the “true” Δ_{47} defined by Wang et al. (2004) defines an empirical transfer function (ETF) for each mass spectrometer and associated purification device. This is referred to as a primary reference frame. At Imperial College a primary reference frame was employed over the period 07/05/12 to 13/02/13 on Nina (Figure 2.13) and 12/02/13 to 09/05/13 on Pinta (Figure 2.14) during which time the values of carbonate standards ICM and ETH3 were determined.

Secondary reference frame

The ETF must be continually monitored to account for changes in source scrambling and other analytical effects however the production and measurement of equilibrated gasses is

awkward and time-consuming. Measuring reference gases also deviates from the established principle of measuring a standard as similar as possible to the sample (in this case, a carbonate). It is therefore common to develop a secondary reference frame based on carbonate standards defining a second ETF, which can be monitored over time. The ‘accepted’ Δ_{47} values of carbonate standards was calculated within the primary reference frame using the ETF defined by equilibrated gas measurements. The ‘true’ values of ICM and ETH3 were estimated, based on equilibrated gases run over the period 07/05/12 to 13/02/13 on mass spectrometers Nina and Pinta and re-calculated using the “crunching” parameters of Brandt et al. (2012). The “true” value of ETH2 has been subsequently determined as 0.3‰. Equilibrated gasses are occasionally run, such as EGS30 on Pinta 03/03/2017 to 21/08/2017 (Figure 14), to verify accepted values and test the validity of the secondary transfer function over the measurement period.

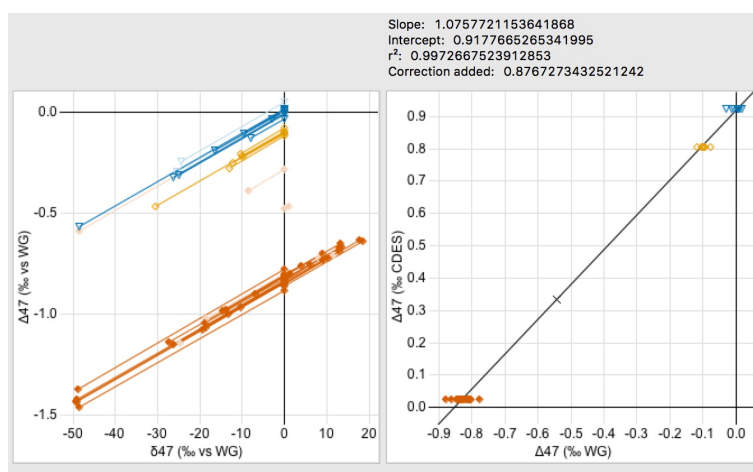


Figure 2.13 Reference frame and empirical transfer function (ETF) on mass spectrometer Nina 07/05/12 to 13/02/13

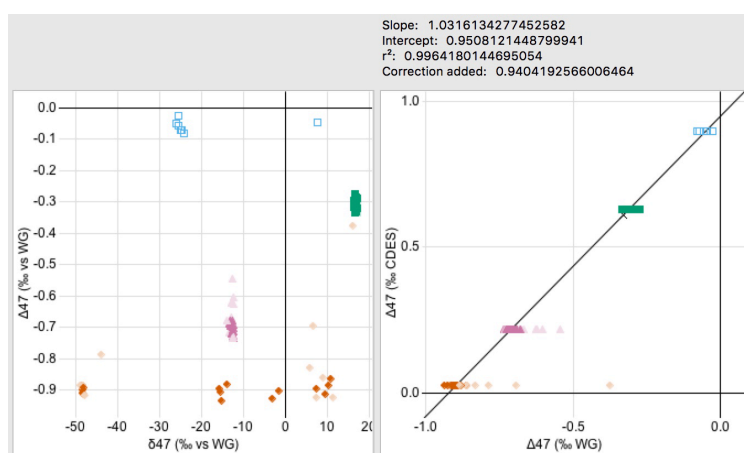


Figure 2.14 Reference frame and empirical transfer function (ETF) on mass spectrometer Pinta 03/03/2017 to 21/08/2017

The accepted or “true” Δ_{47} values of equilibrated gasses at the equilibration temperatures we used, and the carbonate standards we determined are shown in Table 1.

Table 1 Standards used in primary and secondary reference frame correction at ICL

Standard	Description	Value	Reference
ICM	Carrara Marble from Italy heated to approximately 300°C (Molli et al., 2000).	0.409	Davies and John 2017
ETH2	Heated Carbonate for inter laboratory comparison supplied by Stefano Bernasconi (ETH Zurich)	0.3	Independently determined based on 1° reference frame
ETH3	Cretaceous chalk supplied Stefano Bernasconi (ETH Zurich) for interlaboratory comparison	0.713	Independently determined based on 1° reference frame
HGS (1000°C)	Heated gas, heated to 1000°C for 4 hours	0.027	Wang et al. (2004)
EGS (30°C)	CO ₂ water equilibrated at 30°C	0.899	Wang et al. (2004)
ETH1 (used only on IBEX)	Heated Carbonate for inter laboratory comparison supplied by Stefano Bernasconi (ETH Zurich)	0.3	Independently determined from ETH 2, ICM and HG based on 4 IBEX reference frames
ETH4 (used only on IBEX)	Carbonate supplied Stefano Bernasconi (ETH Zurich) for inter-laboratory comparison	0.558	Based on 4 ibex reference frames (n = 34). Similar to Schauer et al. 2016

2.3.5 Correction for acid fractionation

In the early years of the clumped isotope measurement, acid digestion was carried by 18-24 hour reaction at 25°C (Ghosh et al., 2006; Came et al., 2007; Guo and Eiler, 2007). However to speed up the analytical process it is now common practice to raise acid digestion temperature to 90°C thus shortening digestion time to around 10 minutes for calcite (Dennis and Schrag, 2010). Acid digestion at 90°C also results in increased Δ_{47} reproducibility (Wacker et al., 2013). Variation in digestion temperature however has a measureable effect on the relative abundance of ¹³C-¹⁸O of CO₂ evolved from isotopically identical carbonates (Wacker et al., 2013). The variation in acid fractionation factor (AFF) was theoretically estimated by Guo et al. (2009), acid digestion fractionation (‰) increasing with decreased temperature for multiply substituted species Δ_{47} , Δ_{48} and Δ_{49} . The theoretical Δ_{47AFF} 25-90°C of 0.069‰ (Guo et al., 2009) was used for data processed at Imperial College until prior to a switch to the fractionation factor of Defliese et al. (2015) on 22/06/2017. Defliese et al. (2015) define a single dependant acid fractionation relationship:

$$1000\ln\alpha_{CO_2(Acid)-\Delta_{47}} = \frac{(0.022434 \pm 0.001490) \times 10^6}{T^2} - (0.2524 \pm 0.0168)$$

(Equation 2.19)

independent of mineralogy where $T \propto$ is phosphoric acid fractionation factor and T is degrees in Kelvin giving a $\Delta_{47}^{AFF\ 25-90^\circ C}$ of 0.082‰. Data is presented $\Delta_{47}^{CDES25^\circ C}$ where by adding the AFF 0.082‰ for carbonates acid digested at 90°C and 0.062‰ for the limited number of replicates reprocessed from the original Kluge calibration (Kluge et al., 2015) acid digested at 70°C.

2.4 Metrics of contamination

Δ_{48} may be calculated in an analogous fashion to Δ_{47} (see section 2.2.3). The offset in the Δ_{48} value of a sample from δ_{48} vs. Δ_{48} heated gas regression lines was used to detect the presence of contaminants that may fall within the range of the mass 47 isotopologue of carbon dioxide (Huntington et al. 2009; Wacker et al. 2013). Heated gases are considered nominally pure as the heating procedure is thought to degrade most contaminant molecules present. Heated gases that are offset from a δ_{48} vs. Δ_{48} least squares regression, possibly as a result of SO_2 or SO_3 contamination, are disabled and not included in the regression. Those samples with a Δ_{48} offset more than 1.5 ‰ outside the range of the heated gas lines were deemed contaminated and unsuitable for use in analyses. An occasionally used measure of the degree of contamination in a sample is the 49 parameter (John and Bowen, 2016). This is calculated based on the difference in the m/z 49 measured signal between the sample and reference gases. At Imperial College we use the nominal 49 parameter value of 0.2 above which samples are considered contaminated. This is further discussed in chapter 3.

2.5 Clumped Isotope Calibration

The choice of inorganic calibration with which to compare biogenic datasets is an important one. As a “vital effect” reflects the difference in isotopic composition between a biologically produced carbonate and inorganic carbonate produced at thermodynamic equilibrium under the same conditions we must have confidence in the Δ_{47} -temperature relationship for inorganic calcite.

In order to accurately determine non-equilibrium effects in biogenic carbonate comparison with carbonate produced in thermodynamic lattice equilibrium, independent of the solution chemistry we must consider the cause of differences in calibrations produced using synthetic carbonate precipitates. Causes of these differences include precipitation method, discrepancies acid fractionation and analytical artifacts relating to CO_2 purification and IRMS.

The first inorganic Δ_{47} -temperature calibrations of the clumped isotope thermometer (Ghosh et al., 2006; Dennis and Schrag, 2010) differ significantly in temperature sensitivity. The Δ_{47} -temperature slope of the original Ghosh et al. (2006) calibration ($m = 0.0636 \times 10^6/T^2$) is much steeper than that of Dennis et al. (2010) ($m = 0.0362 \times 10^6/T^2$). Following inorganic calibrations fall within these two end members but are grouped widely into two groups, those with a steep slope reflecting the original Ghosh et al. (2006) calibration (e.g. Zaarur et al., 2013) and those with a shallower slope following both Dennis et al. (2011) and the theoretical calibration of Guo et al. (2009) (e.g. Fernandez et al., 2014; Tang et al., 2014; Defliese et al., 2015).

Fernandez et al. (2017) illustrate how the number of replicates, sample number and temperature range exert a strong temperature control on the robustness of the slope and precision of the clumped isotope calibration. To determine the presence of “vital effects” in biogenic carbonate measured the Δ_{47} of biogenic carbonate with the combined is thus compared with inorganic calibrations of Kluge et al. (2015) and Kelson et al. (2017). These calibrations have been selected as they encompass multiple precipitation methods, cover a wide temperature range and incorporate a high density of measurements in the low temperature range (Fig. 2.15). Further details of the combined inorganic equation used in this study are provided in chapter 4, section 4.6.1.

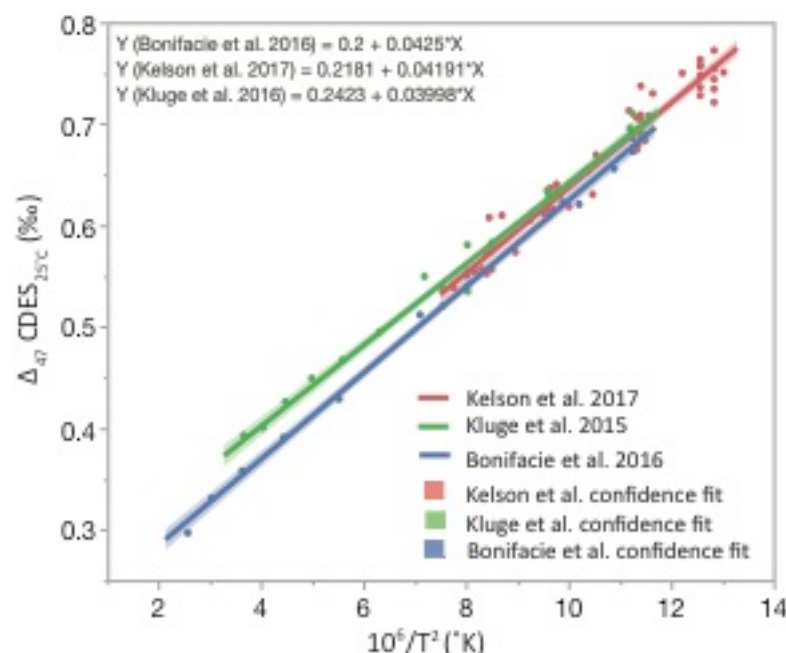


Figure 2.15 Kelson et al. (2017) and Kluge et al. (2015) calibrations re-processed using the Brand et al. (2010) ^{17}O correction parameters. The T - Δ_{47} calibration of (Bonifacie et al., 2016) for dolomite is also shown however is not incorporated into the combined inorganic calibration owing to the deviation from Kluge et al. (2015) at high temperature.

2.6 Statistical Methods

Statistical analysis was carried out using the software JMP® version 13.2.0. Further details of statistical methods are described in **chapter 4**. In one way-ANOVA tests we report DF(Groups), DF(Error), F-values and p-values following APA style (Association, 1994). DF(Groups) gives the between group variation calculated as k-1, DF(Error) gives the within group variation N-k. The F-Statistic gives variation between sample means/variation within samples thus comparing the variability between groups to the variability within groups. Low F-values indicate close group means however in order to interpret F-values we present the p-value. The p-value gives the probability of obtaining a value above the F-value on the F-distribution, which follows the ratio of between group variability to within group variability assuming the null hypothesis is true. P-values of >0.05 or >0.01 enable us to reject the null hypothesis at the confidence level of 95% or 99% respectively.

When assessing suitability of one-way ANOVA in analyses we must consider the effect of sample size. The “effect size” quantifies the difference between groups emphasizing the size of the difference free of sample size effects. We can calculate the effect size between groups η^2 .

$$\eta^2 = SS(\text{Between groups})/SS(\text{Total}) \quad (\text{Equation 2.20})$$

where SS(Between groups) is the sum of squares for the effect of the independent variable and SS(Total) is the total sum of squares (Richardson, 2011).

In one-way ANOVA analyses we, on average, test 5-6 groups, each representing a skeletal element with 3-4 replicates in each group. Effect size varies from 0.2 to 0.29, a medium effect size (Cohen, 1992). Power values for ANOVA analyses in this study are <0.2 at a significance level of 0.05. Low power values indicates a high probability of type II errors manifested and less than a 0.2 chance of rejecting the null hypothesis. We however present the results of these statistical tests as a first step to more quantitative analysis of clumped datasets.

The coefficient of determination (R^2) (Equation 2) is used to quantify the significance of association between two continuous variables x and y such as temperature and Δ_{47} . Assessment of correlation is important in identifying covariates.

$$R^2 = \left(\frac{n(\Sigma xy) - (\Sigma x)(\Sigma y)}{\sqrt{[n\Sigma x^2 - (\Sigma x)^2][n\Sigma y^2 - (\Sigma y)^2]}} \right)^2 \quad (\text{Equation 2.21})$$

ANCOVA is a parametric test assuming independent observations and that the covariate and dependent variable are linearly related with equal slope. In the case of this work, this implies testing the difference in Δ_{47} -temperature calibrations where inorganic and biogenic calibrations form independent categorical variables, Δ_{47} is the dependent variable and temperature is the covariate. As in one way ANOVA analyses we report DF(Groups), DF(Error), F-values and p-values.

To further verify ANCOVA results we carry out student t-tests on least squared mean difference between the two groups tested. As for ANOVA can test for effect size

$$d = m_a - m_b / \sigma \quad (\text{Equation 2.22})$$

where d is effect size m_a is group a mean m_b is group b mean and σ is pooled standard deviation $\sigma = \sqrt{[(\sigma_a - \sigma_b)/2]}$.

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Chapter 3: Reducing contamination parameters for clumped isotope analysis: The effect of lowering Porapak™ Q trap temperature to below -50°C

ABSTRACT

RATIONALE: Carbonate clumped isotope thermometry examines the thermodynamic preference of ^{13}C - ^{18}O bonds to form within the carbonate crystal lattice. The $^{13}\text{C}^{18}\text{O}^{16}\text{O}$ isotopologue in analyte CO_2 has a natural abundance of 44.4ppm necessitating stringent purification procedures to remove contaminant molecules that may produce significant isobaric effects within range of the mass 47 isotopologue. Strict purifications of analyte CO_2 are thus required as well as reliable contamination indicators.

METHODS: CO_2 purification was carried out by vacuum cryogenic purification through the static trap packed with Porapak™ Q (PPQ). The correlation between mass excesses on m/z 47, 48 and 49 in CO_2 produced by acid digestion of 12 natural samples was measured by IRMS. CO_2 from two contaminated carbonate samples was then purified at PPQ trap temperatures between -25°C and -65°C and measured by IRMS to determine changes in mass excesses on m/z 47, 48 and 49. Finally carbonate standards, Carrara Marble (CM) and ETH3, were purified at PPQ trap temperatures -35°C and -60°C to identify isotopic fractionation associated with lowering trap temperature.

RESULTS: The correlation between mass excesses on m/z 47, 48 and 49 is determined to be sample dependent. Lowering PPQ trap temperature to -60°C has a 78% success rate in decreasing $\Delta_{48\text{offset}}$, a measure of sample contamination, to within an acceptable range (<1.5‰). Lowering PPQ temperature in purification of CM and ETH3 is associated with decreases in $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ as a result of isotopic fractionation. We demonstrate that we can correct for fractionation at trap temperature of -60°C.

CONCLUSIONS: Lowering the temperature of the Porapak Q trap to -60° C results in improved sample cleaning. It is possible to correct for fractionation in $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ lower PPQ temperatures using identically prepared standards. This result has important connotations for laboratories using similar sample preparation methods.

1) INTRODUCTION

Clumped isotope geochemistry is the study of naturally occurring molecules containing two or more rare isotopes. (Eiler, 2007) Carbonate clumped isotope analysis specifically examines the extent to which ^{13}C and ^{18}O bond together in the carbonate crystalline lattice. The formation of ^{13}C - ^{18}O bonds is based on the temperature dependent homogenous isotope exchange equilibrium: $\text{M}^{13}\text{C}^{16}\text{O}_3 + \text{M}^{12}\text{C}^{18}\text{O}^{16}\text{O}_2 \rightleftharpoons \text{M}^{13}\text{C}^{18}\text{O}^{16}\text{O}_2 + \text{M}^{12}\text{C}^{16}\text{O}_3$, decreasing temperature favoring the formation of $\text{M}^{13}\text{C}^{18}\text{O}^{16}\text{O}_2$ (where M is a metal such as Ca)(Schauble et al., 2006). As current methods of analysis require a gas analyte, the carbonate is acid digested to produce CO_2 in which the abundance of the $^{13}\text{C}^{18}\text{O}^{16}\text{O}$ isotopologue is proportional to the extent of ^{13}C - ^{18}O ordering in the carbonate. The excess of $^{13}\text{C}^{18}\text{O}^{16}\text{O}$ relative to a stochastic distribution, in which all isotopes are equally distributed among isotopologues, is then measured and expressed using the metric Δ_{47} (Eiler and Schauble, 2004) defined as:

$$\Delta_{47} = \left(\frac{R^{47}}{R^{47*}} - 1 \right) - \left(\frac{R^{46}}{R^{46*}} - 1 \right) - \left(\frac{R^{45}}{R^{45*}} - 1 \right) \times 1000$$

(Equation 3.1)

Where $R^i = m_i/m_{44}$ and R^* gives the abundance of each isotopologue of a gas with the same bulk isotopic composition as R but with a stochastic distribution (Affek and Eiler, 2006).

The $^{13}\text{C}^{18}\text{O}^{16}\text{O}$ isotopologue in analyte CO_2 has a natural abundance of only 44.4 ppm at 25°C , thus small isobaric effects on mass 47 can have a significant impact on calculated carbonate formation temperature. Short chain hydrocarbons, halocarbons and sulfide gases are all potential sources of contamination (Ghosh et al., 2006; Eagle et al., 2010; Tripathi et al., 2010). Gas molecules such as these may undergo fragmentation and/or recombination reactions in the source of the mass spectrometer producing significant isobaric interferences within the mass range 44-49 (Huntington et al., 2009). The natural abundance of isotopologues of CO_2 with a mass of 49 is undetectable by mass spectrometry, while the natural abundance on mass 48 in atmospheric CO_2 at 25°C is 3.98 ppm. The presence of a measurable signal on masses 48 and 49 has thus been used as a sign of contamination on all masses, including on mass 47 (Eiler and Schauble, 2004).

The application of the clumped isotope method to an increasing variety of geological problems has led to an expansion in the types of samples on which clumped isotopic analysis has been performed. This includes meteorites (Guo and Eiler, 2007; Halevy et al., 2011)

cataclasites (Swanson et al., 2012) and concretions (Loyd et al., 2012; Dale et al., 2014). Materials such as these often contain high concentrations of organic compounds, which may be volatilized on acid digestion to produce short-chain organic molecules. Elemental sulfur and sulfur compounds may also be volatilized to produce sulfide gases.

Purification of carbon dioxide gas for clumped isotopic analysis is increasingly carried out using automated sample devices such as the modified Thermo Scientific Kiel IV (Schmid and Bernasconi, 2010) and small sample preparation inlets (Petersen and Schrag, 2014). These facilitate the removal of contaminants from evolved carbon dioxide by cryogenic purification through the stable phase Porapak Q (hereafter “PPQ”), without a helium carrier gas, following a commonly used method (Dennis and Schrag, 2010). This method, though easier to implement than gas chromatographic purification with a helium carrier remains, with the exception of two studies (Petersen et al., 2015; Wang et al., 2016) relatively untested with respect to optimum operating temperature and memory effects of the PPQ trap.

The goal of this paper is to test the efficiency of the CO₂ purification procedures for clumped isotopic analysis in which CO₂ is passed through a PPQ column without carrier gas by cryogenic trapping. The effect of PPQ trap temperature on gas yield, contaminant removal, Δ_{47} and bulk isotopic values has been previously illustrated (Petersen et al., 2015). This study however focused on standards with low Δ_{48} offset values at PPQ trap temperatures between -10°C and -40°C. By further lowering the temperature at which the PPQ trap is held to below -50°C we attempted to enhance separation between CO₂ and contaminant gases with longer retention time on the stable phase Porapak Q, such as halocarbons and hydrocarbons, resulting in more efficient CO₂ purification. In contrast to previous studies, (Petersen et al., 2015; Wang et al., 2016) we address purification of samples with problematically high mass excesses on m/z 48 and 49 manifested in high Δ_{48} offset and 49 parameter values (John and Bowen, 2016). This is achieved by the measurement of 2 contaminated samples at 9 different PPQ temperatures between -65°C and -25°C. This also enables review of the contamination parameters Δ_{48} offset and 49 parameter used to quantify the “cleanliness” of evolved CO₂ gas. Uncontaminated standards, used as control experiments, test whether trap temperature introduces analytical bias.

This study, the first to test purification at PPQ trap temperatures below -40°C, illustrates effective, achievable improvements in sample purification procedures that may be applied to both manual and automated sample preparation procedures.

2) METHODS

2.1 Samples and standards used

Sample preparation and isotopic analyses were carried out in the Qatar Stable Isotope Laboratory at Imperial College London. In preparation for analysis, all samples were finely powdered using a dental drill. Typical sample sizes used for analysis were in the range of 5-10 mg.

Heated gases and carbonate standard were measured alongside samples to allow for data correction and conversion to the absolute reference frame (Dennis et al., 2011). Heated gasses are prepared measured as described in **chapter 2**.

The carbonate standards used in this study were:

ETH3: Inter-laboratory calcite standard with an accepted Δ_{47} value of 0.705‰, (Meckler et al., 2014) used to project values into the absolute reference frame (Dennis et al., 2011).

Carrara Marble (CM): Pure calcite standard from Carrara, Italy which has been metamorphosed to greenschist facies (~360°C) during the Alpine orogeny (Molli et al., 2000). Average Δ_{47} of carrara marble replicates measured at standard PPQ temperature of -35°C is 0.388 ± 0.005 (n = 74).

The carbonate samples used in this study were:

DDOL1-6: Dolomite samples exhibiting high Δ_{48} offset and 49 parameter values before and after treatment of the powdered sample with hydrogen peroxide and multiple CO₂ purification attempts. (Heim, 2011)

DCM1: ~5 mg Carrara marble standard mixed with up to 2 mg of commercial gypsum powder (CaSO₄·2H₂O). Gypsum was obtained from the Sorbas Basin, Spain and had dark inclusions of organic matter.

DCM2: Carrara marble standard mixed with an evaporitic anhydrite (CaSO₄) rich sample from the Jurassic Weald anhydrite in the UK. (Abbott et al., 2016) Anyhdrite was mixed with Carrara marble in order to generate an adequate CO₂ yield on acid digestion for analysis. Samples typically consisted of 5 mg of anhydrite and 5 mg of Carrara marble.

2.2 Purification of CO₂ through the PPQ trap

Acid digestion and manual CO₂ purification was carried out as described in **chapter 2**. The silver trap was not cleaned or exchanged during the course of this study thus its effect is assumed constant in all analyses.

2.3 IRMS analysis

Two Thermo MAT 253 gas source mass spectrometers (Thermo Instruments Bremen, Germany) 'Nina' and 'Pinta' were used to measure sample isotopic composition. IRMS measurement procedure is described in **chapter 2**. A typical acquisition time is 20 minutes corresponding to a total analysis time of 2.5-3 h. The working reference gases were both CO₂ standards from Oztech, with $\delta^{13}\text{C}_{\text{VPDB}} -3.62\text{‰}$, $\delta^{18}\text{O}_{\text{VSMOW}} 25.09\text{‰}$ for Pinta and $\delta^{13}\text{C}_{\text{VPDB}} -3.63\text{‰}$, $\delta^{18}\text{O}_{\text{VSMOW}} 25.02\text{‰}$ for Nina.

2.4 Data Correction Procedures

$\Delta_{47\text{raw}}$, $\Delta_{48\text{raw}}$, δ_{47} , δ_{48} , δ_{49} , $\delta^{18}\text{O}_{\text{raw}}$ and $\delta^{13}\text{C}_{\text{raw}}$ values were calculated using the computer software program Easotope (John and Bowen, 2016) as described in **chapter 2**. Beam intensities in millivolts are converted to δ^i values. δ^i is a bulk value, correlated with the $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ values of the carbonate sample and the reference gas used for the analysis (Equation 2) (Affek and Eiler, 2006).

$$\delta_{47} = \left(\frac{R^{47}_{\text{sample}}}{R^{47}_{\text{reference}}} - 1 \right) \times 1000$$

(Equation 3.2)

$\Delta_{47\text{raw}}$ is calculated as described in **chapter 2**. The isotopic parameters of Gonfiantini et al. (1995) are used to define ($^{13}\text{C}/^{12}\text{C}$) ratio of VPDB, the ($^{17}\text{O}/^{16}\text{O}$) and ($^{18}\text{O}/^{16}\text{O}$) ratios of VSMOW and the slope of the triple oxygen isotope line (λ). The difference between calculated Δ_{47} CDES, δ_{final} , $\delta^{13}\text{C}_{\text{final}}$ and $\Delta_{48\text{offset}}$ values using the isotopic parameters of Gonfiantini et al. (1995) and those calculated using the parameters of Brand et al. (2010) for sample DDOL1 as an example are minimal with both values within range of counting statistics ($<0.01\text{‰}$) in Δ_{47} CDES and <0.34 in $\Delta_{48\text{offset}}$.

In this study, the raw data for Δ_{47} is corrected in three steps unless otherwise discussed within the text. These corrections were also performed using Easotope. (John and Bowen, 2016) First, in order to correct for non-linearity of the mass spectrometer, heated gases with different bulk isotopic compositions, are measured and normalized by projecting all Δ_{47} measurements to a δ^{47} of 0 (Huntington et al., 2009). Δ_{47} measurements corrected for non-linearity are then translated into the absolute reference frame using standards ETH3, Carrara Marble and heated gasses (Dennis et al. 2011). Δ_{47} is corrected for acid fractionation by adding the acid fractionation factor 0.092‰ (Henkes et al., 2013) and $\delta^{18}\text{O}$ values are corrected for phosphoric acid digestion at 90°C using acid fractionation factors of 1.00930‰ for calcite (Kim and O'Neil, 1997) and 1.00813‰ for dolomite. (Rosenbaum and Sheppard, 1986)

2.5 Metrics of Contamination

Δ_{48} may be calculated in an analogous fashion to Δ_{47} (Huntington et al., 2009). The offset in a sample's Δ_{48} from δ^{48} vs. Δ_{48} heated gas regression lines is used to detect the presence of contaminants that may fall within the range of the mass 47 isotopologue of carbon dioxide (Huntington et al., 2009). Heated gases are considered nominally pure as the heating procedure is thought to degrade most contaminant molecules present. Heated gases that show poor correlation with δ^{48} vs. Δ_{48} regression, possibly as a result of SO_2 or SO_3 contamination, are disabled and not included in the regression. Those samples with Δ_{48} offset more than 1.5 ‰ outside the range of heated gas lines are deemed contaminated and unsuitable for use in analyses. An occasionally used measure of the degree of contamination in a sample is the 49 parameter. This is calculated based on the difference in m/z 49 measured between the sample and reference gases. (John and Bowen, 2016)

3) RESULTS

3.1 Impacts of contamination on Δ_{47} values

To test the effect of increasing contamination on sample Δ_{47} , up to 45 wt% powdered gypsum was added to a Carrara Marble standard (Figure 3.1). In these experiments PPQ trap temperature was kept constant at -35°C. Samples were acid reacted, purified and measured in order of increasing gypsum concentration to mitigate potential memory effects of either the silver or PPQ trap. The mean Δ_{47} of contaminated DCM1 is 0.04‰ greater than the mean of uncontaminated standards. Also important to note is the increase in external error of the measurement on the addition of gypsum CM1: standard deviation values are up to 0.117‰ (1

SD) for contaminated samples in comparison with a standard deviation of 0.04‰ for uncontaminated samples. There is however no significant correlation between gypsum weight added, Δ_{47} and Δ_{48} offset.

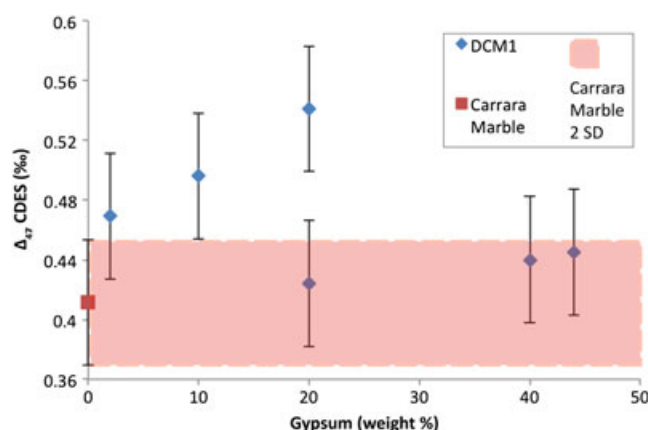


Figure 3.1 Offset in Δ_{47} reference frame corrected (CDES) with additional weight percentage gypsum added to single replicates of Carrara marble. The red box represents the 95% confidence interval in Δ_{47} CDES values of uncontaminated Carrara marble standards measured over the period 1 October 2013 to 1 November 2014

To ensure there is no decrease in the efficiency of the silver wool and PPQ trap in reducing CO_2 contamination over time we monitored the $\Delta_{48\text{offset}}$ of standards (Figure 3.2). No change in Δ_{48} offset of standards over time indicates that purification of the sample gas using both the silver trap and PPQ trap remain consistent under the same analytical conditions throughout the study. Further determination of the effects altering the silver trap is however outside of the scope of this study. We regard the treatment of sample gas using silver wool as a constant and focus on the additional step of purification using the PPQ trap.

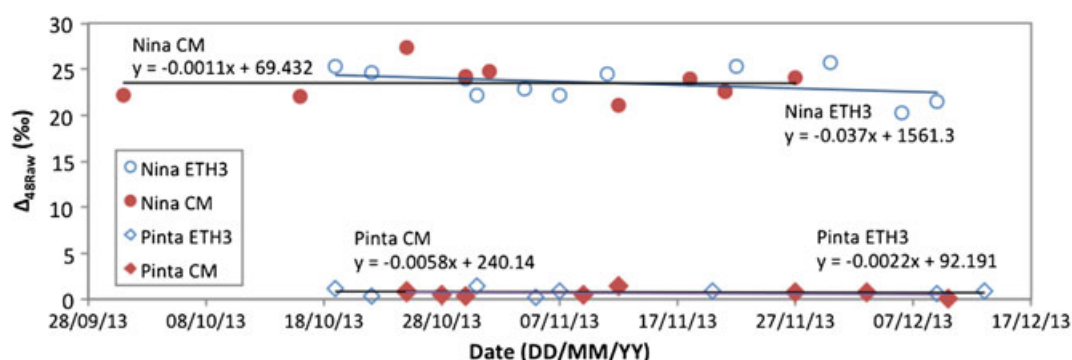


Figure 3.2 Change in $\Delta_{48\text{offset}}$ of Carrara marble and ETH3 standards over the time period 28 September 2103 to 17 December 2013

DDOL samples measured show little correlation between Δ_{48} offset and Δ_{47} excesses (Figure 3.3a). Variability in the Δ_{47} measurement indeed remains high especially for samples DDOL6 and DDOL4, even under the Δ_{48} threshold value of 1.5‰ below which samples are considered

free of contamination. The lack of clear correlation between Δ_{48} offset and Δ_{47} is also reflected in modern shells measured (Figure 3.3c). All modern shells measured with Δ_{48} offset $<1.5\text{‰}$ however have Δ_{47} CDES values within the expected range based on growth temperature using the calibration of Kluge et al. (2015). In contrast over 50% of measurements Δ_{48} offset $>1.5\text{‰}$ show erroneously high Δ_{47} .

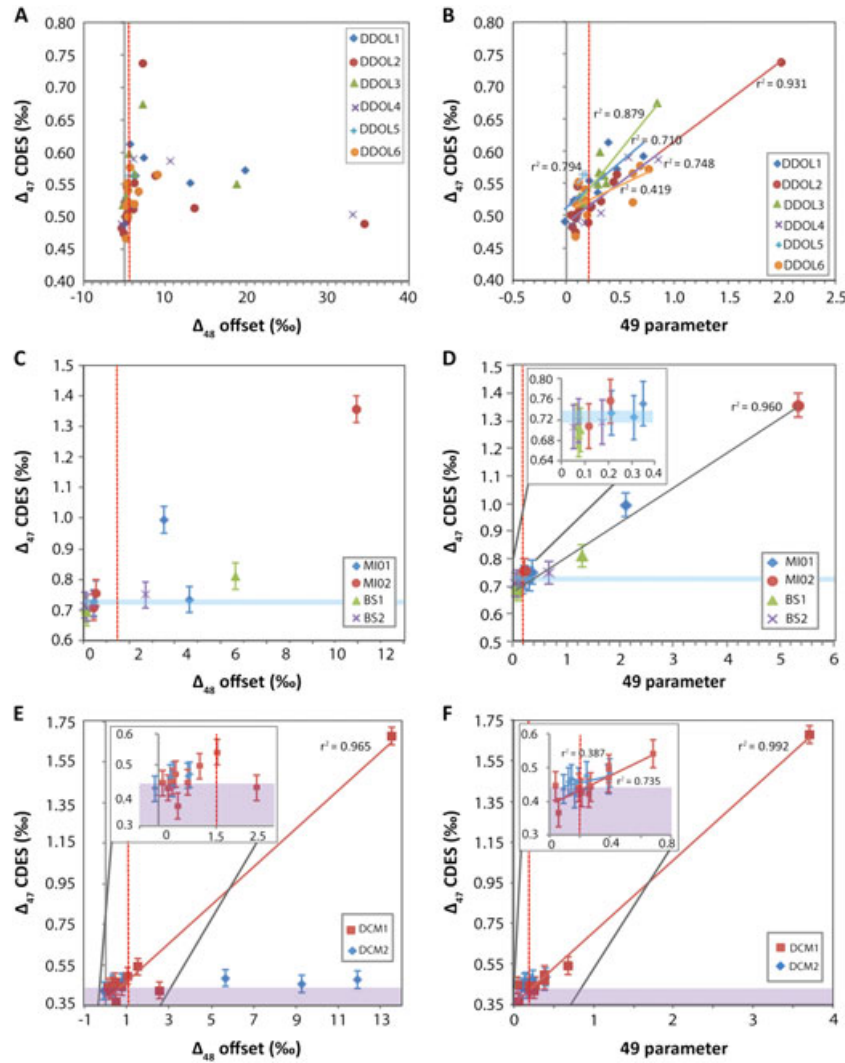


Figure 3.3 Correlation between A) Δ_{48} offset and Δ_{47} CDES for contaminated dolomite (DDOL) samples; B) 49 parameter and Δ_{47} CDES for contaminated dolomite (DDOL) samples; C) Δ_{48} offset and Δ_{47} CDES for modern shell samples; D) 49 parameter and Δ_{47} CDES for modern shell samples; E) Δ_{48} offset and Δ_{47} CDES for contaminated Carrara marble samples DCM1 and DCM2; and F) 49 parameter and Δ_{47} CDES for contaminated Carrara marble samples DCM1 and DCM2

Δ_{48} offset values of DCM1, purified at PPQ trap temperature -35°C , are well correlated with Δ_{47} (Figure 3.3e). The Δ_{47} of DCM1 a Δ_{48} offset of 1.52‰ is 0.518 at giving a temperature of formation of 107°C , 146°C lower than the expected temperature of 253°C (Kluge et al, 2015). Furthermore DCM1 samples with Δ_{48} offset values $<1.5\text{‰}$ exhibit Δ_{47} values within 1 SE of the

expected value of uncontaminated standards 0.389 ± 0.05 ($n = 74$). Higher Δ_{48} offset values in sample DCM2 however do not correspond to higher Δ_{47} measurements.

We observe positive correlation between sample Δ_{47} and the 49 parameter in all samples measured (Figure 3.3). In dolomite samples DDOL1-6 there is good correlation between the 49 parameter and Δ_{47} , 5 of 6 samples have an $r^2 > 0.7$ (Figure 3.3b). This is evident for sample DDOL1 in which measurements with a 49 parameter > 0.19 have Δ_{47} values of between 0.49‰ and 0.67‰ while for replicates with a 49 parameter < 0.19 Δ_{47} varies between 0.39‰ and 0.42‰. The difference in Δ_{47} values corresponds to a difference of 49°C based on Kluge et al. (2015)

There is also excellent correlation between the 49 parameter and Δ_{47} in the modern shells measured, $r^2 = 0.960$. The correlation between 49 parameter and Δ_{47} sample replicates for modern shells is consistently good with all samples with $r^2 > 0.93$ (Figure 3.3d). 49 parameter values of DCM1 are also well correlated with measured excesses on mass 47 while samples of DCM2 are weakly correlated with Δ_{47} (Figure 3.3f).

The dependence of mass 47 excesses on 49 parameter values is however variable. The linear regression between 49 parameter and Δ_{47} of DDOL2 has a gradient of 0.21 over twice as steep as that of DCM1 (0.09) (Figure 3.3b,f).

3.2 Effect of PPQ trap temperature on $\Delta_{48\text{offset}}$ and 49 parameter values

DDOL1 was purified at 6 PPQ trap temperatures between -25°C and -65°C (Figure 3.4). DDOL1 exhibits an average Δ_{48} offset of 18.30‰ when purified with the PPQ trap at -35°C. At lower trap temperatures a decrease in average Δ_{48} offset is observed, falling to 0.24‰ at -65°C. This is below the Δ_{48} offset threshold of 1.5‰ used at Imperial College London below which samples are generally considered clean and suitable for use in analysis. This is however not accompanied by a notable decrease in Δ_{47} CDES (‰) (Figure 3.4). At -65°C a decrease in the standard deviation of Δ_{48} measurement to 0.218‰ is also observed. This is much closer to the average “background” variability in Δ_{48} exhibited by three consecutive Carrara marble standard replicates of 0.017 ± 0.002 ‰.

DCM2 was also purified at 7 PPQ trap temperatures between -25°C and -65°C. As observed for sample DDOL1 there is a decrease in Δ_{48} offset and 49 parameter with decreased PPQ trap

temperature (Figure 3.4). Below -50°C , Δ_{48} offset values are $>1.5^{\circ}\text{C}$ and 49 parameter values are >0.2 . In DCM1 the decrease in Δ_{48} offset and 49 parameter observed is also accompanied by a decrease in Δ_{47} values. Average Δ_{47} for samples purified at PPQ temperature of -45°C and above is 0.445‰ (excluding the anomalous Δ_{47} measurement at -35°C) while at PPQ temperatures of -55°C and below, average sample Δ_{47} is 0.429‰ . This corresponds to an 11°C lower temperature using the calibration of Kluge et al. (2015), a greater than 10% difference in carbonate formation temperature.

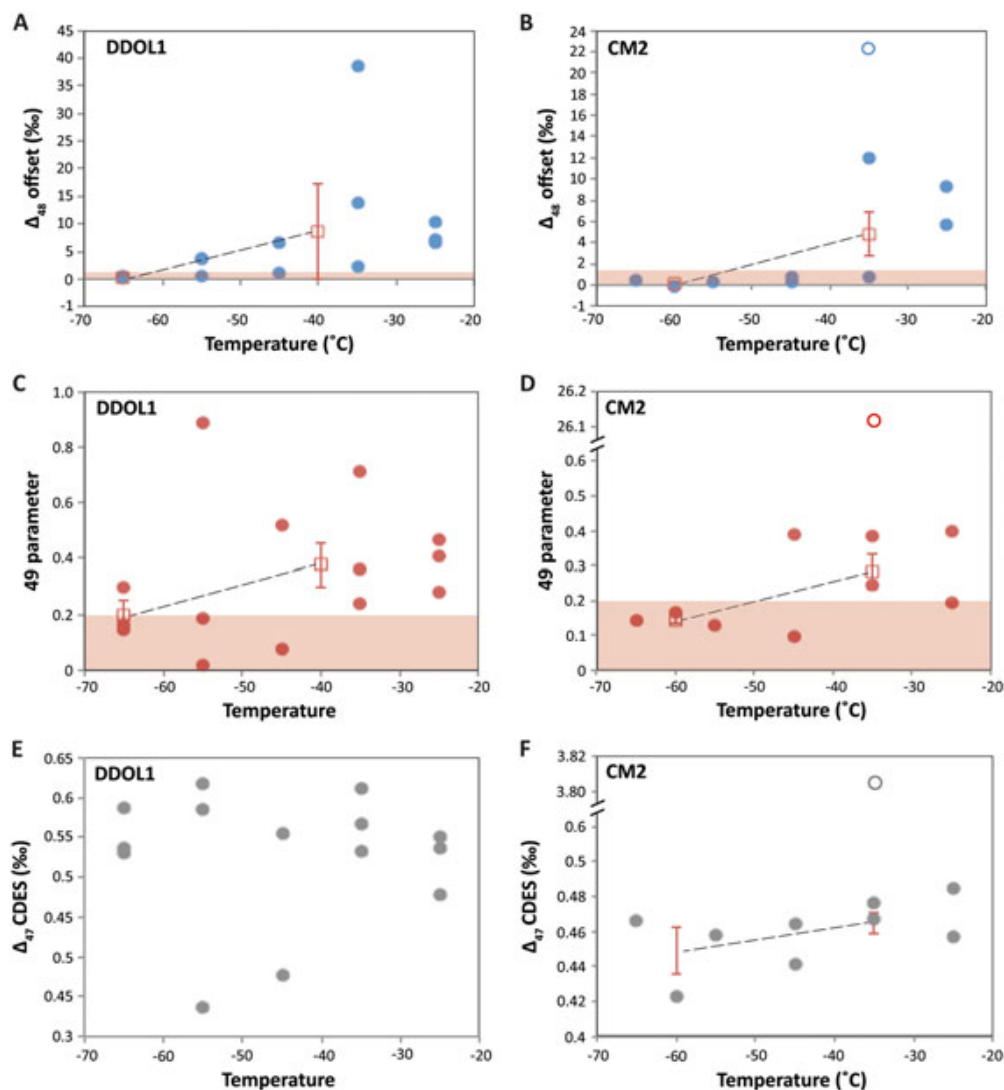


Figure 3.4 Variation in Δ_{48} offset and 49 parameter with PPQ trap temperature measurements of sample DDOL. Red boxes indicates ranges in which the Δ_{48} offset and 49 parameter should fall if the sample is 'uncontaminated' (Δ_{48} offset $< 1.5 \text{‰}$, 49 parameter < 0.2) Open circles represent individual measurements while filled circles denote the mean value (error bars 1 SE)

Over the period ranging from 18 February 2014 to 19 January 2015 samples with Δ_{48} offset values between 0.27‰ and 9.15‰ prepared at a PPQ trap temperature at -35°C were also prepared with the PPQ trap held at -60°C (Figure 3.5). Of these, 33% of samples purified at -35°C have an average Δ_{48} offset greater than 1.5‰ . Following carbon dioxide purification

with the PPQ trap at -60°C , two thirds of samples measured showed lower $\Delta_{48}\text{offset}$ s. Moreover 5 of the 7 samples exhibiting $\Delta_{48}\text{offset}$ values above 1.5‰ when purified at -35°C had Δ_{48} offset values below 1.5‰ when purified at a PPQ temperature of -60°C . When including samples DDOL1 and CM2 measured at PPQ temperatures of -60°C and -35°C in this data set, we conclude that cleaning samples at -60°C results in a 78% success rate for samples that were systematically considered as contaminated when treated at -35°C .

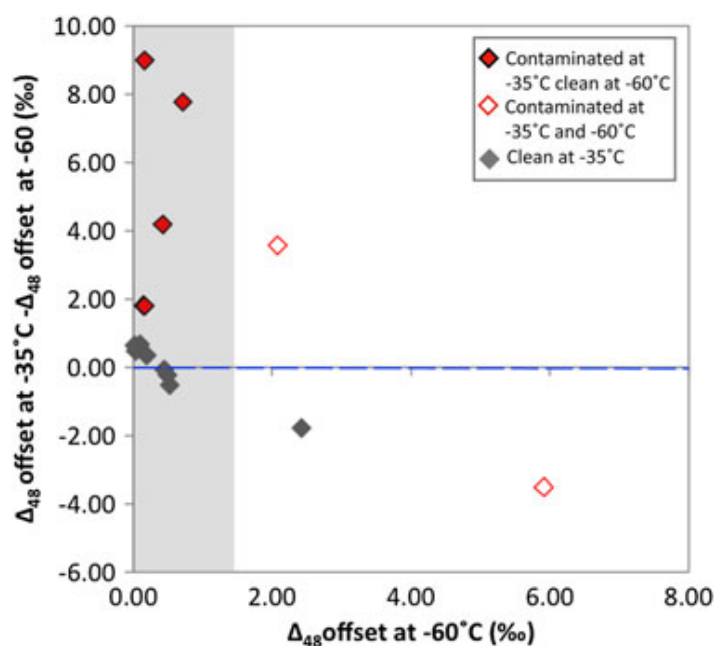


Figure 3.5 $\Delta_{48}\text{offset}$ values of samples purified at -60°C vs change in $\Delta_{48}\text{offset}$ when the PPQ trap temperature was lowered from -35°C to -60°C between replicates. Points falling within the grey shaded region indicate that samples are 'uncontaminated' when purified at -60°C ($\Delta_{48}\text{offset}$ is $<1.5\text{‰}$). Points plotting above the blue line show improvement in Δ_{48} offset when purified at a PPQ temperature of -60°C in comparison with at -35°C

3.3 Effect of PPQ trap temperature on $\delta^{18}\text{O}$, $\delta^{13}\text{C}$ and Δ_{47} values

DDOL1 shows a collapse in $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ to lower values on lowering PPQ temperature below -45°C . This is also associated with an increase in the standard deviation in $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ (Figure 3.6). Sample DDOL1 however exhibits significant variation in $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ at all PPQ trap temperatures (up to 3‰ at -35°C and 5‰ at -65°C). This behaviour is typical of a solid solution, i.e. a sample that contains multiple phases of carbonates and does not reproduce very well due to high heterogeneities. We thus conclude that DDOL is not ideal to determine changes in $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ with decreased PPQ trap temperature.

Instead, we focus on the pure CM and ETH3 standards purified at PPQ temperatures of -60°C and -35°C over the period 22 January 2014 to 24 March 2014 were measured on mass spectrometer Nina (Figure 3.7). As standards were run over a 46-day time window it was

necessary to assess changes in non-linearity of the mass spectrometer that may prevent direct comparison of raw Δ_{47} over the duration of the measurement period. We applied a linear correction to $\Delta_{47\text{Raw}}$ of CM and ETH3 to account for drift between the beginning and end of the correction interval (Equation 3):

$$\Delta_{47\text{Corr}} = \Delta_{47\text{Raw}} - \left(\frac{d\Delta_{47\text{Raw}}}{dt} \times t \right) \quad \text{Equation (3.3)}$$

where $\Delta_{47\text{Raw}}$ describes non-linearity corrected Δ_{47} values and $\frac{d\Delta_{47\text{Raw}}}{dt}$ is the slope of the shift in $\Delta_{47\text{Raw}}$ to lower values over the measurement period and t is time elapsed since the initiation of the measurement period. This allows comparison of Δ_{47} of standards from both the beginning and end of the measurement period without the added potential error of translation of Δ_{47} into the absolute reference frame. Correction of $\Delta_{47\text{raw}}$ over the course of the measurement period is illustrated (Figure 3.8).

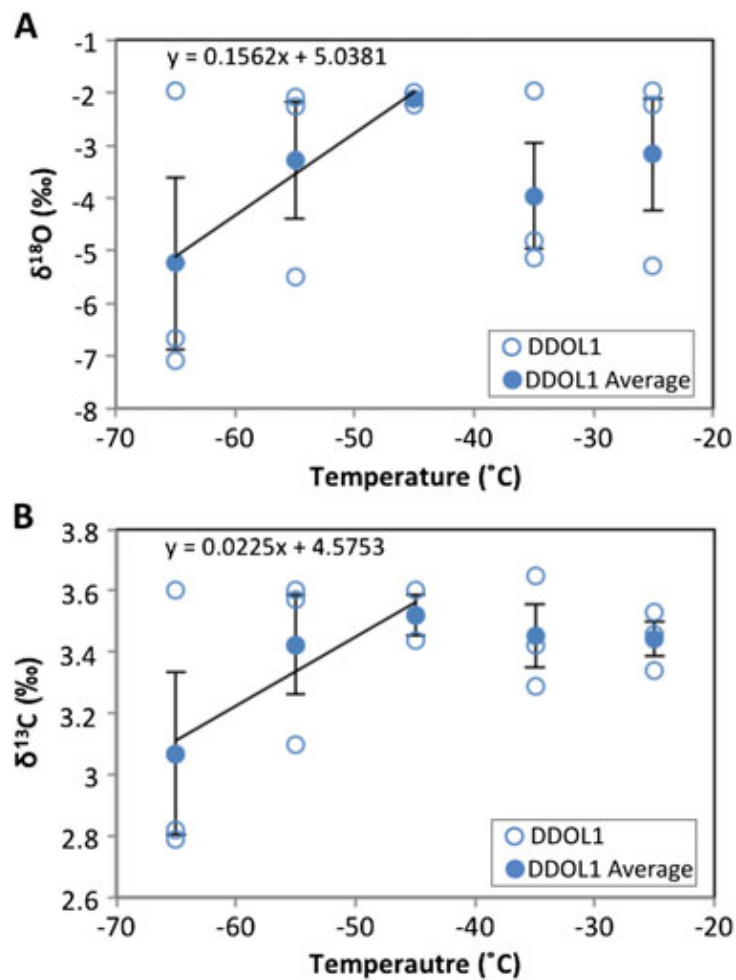


Figure 3.6 Change in $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ values with PPQ trap temperature for sample DDOL. Open circles illustrate individual measurements while filled circles denote the mean value (error bars 1 SE)

The median raw $\delta^{18}\text{O}_{\text{PDB}}$ of CM standards purified at a PPQ temperature of -60°C is -2.61‰ in comparison with a mean of -2.00‰ for standards purified at -35°C . $\delta^{13}\text{C}_{\text{PDB}}$ values for the same CM standards measured have a median of 1.91‰ at -60°C and 2.12‰ at -35°C . This corresponds to a difference of 1.1‰ in back calculated fluid composition ($\delta^{18}\text{O}_{\text{VSMOW}}$) based on an accepted Δ_{47} value of CM of 0.395‰ (Kim and O'Neil, 1997; Kluge et al., 2013). There is an offset in the mean of $\Delta_{47\text{corrected}}$ CM standards of 0.017‰ to lower values when purified at -60 . The inter quartile ranges and standard deviations in $\delta^{18}\text{O}$, $\delta^{13}\text{C}$ and $\Delta_{47\text{Raw}}$ values of CM and ETH3 standards measured during the above period at PPQ trap temperature of -60°C is greater than the standard deviation of those measured at -35°C . For example the interquartile range $\delta^{18}\text{O}$ values of CM standards at -35°C is 0.19‰ while at -60°C over two times greater at 0.46‰ (Figure 3.7)

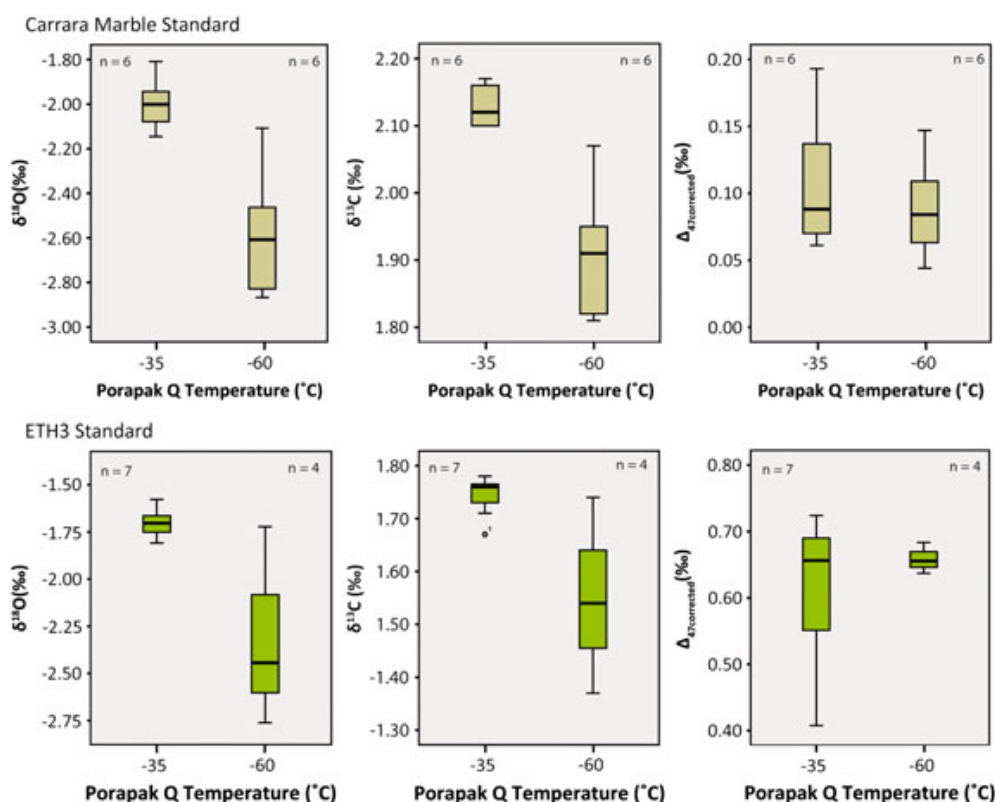


Figure 3.7 Box plots indicating difference in $\delta^{18}\text{O}$, $\delta^{13}\text{C}$ and Δ_{47} values of Carrara marble and ETH3 standards purified at PPQ trap temperatures of -30°C and -60°C . Standards were measured on mass spectrometer Nina over the period 22 January 2014 to 24 March 2014

A histogram of the difference in Δ_{47} CDES between a PPQ trap temperature of -60°C and -35°C (Figure 3.9) illustrates that lowering trap temperature results in lower Δ_{47} values in 53% of samples. The reduction in Δ_{47} is up to 0.11‰ .

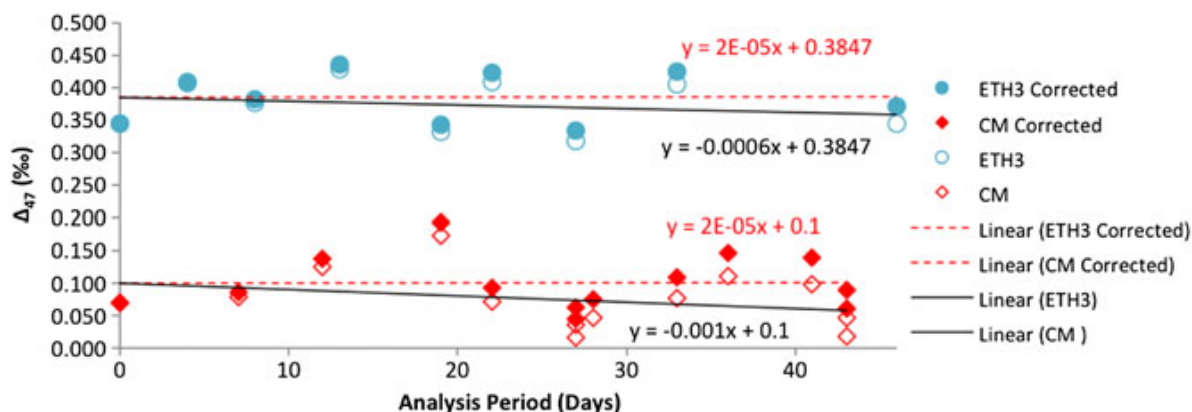


Figure 3.8 Linear correction applied to $\Delta_{47\text{raw}}$ values of CM and ETH3 to account for drift between the beginning and end of the correction interval

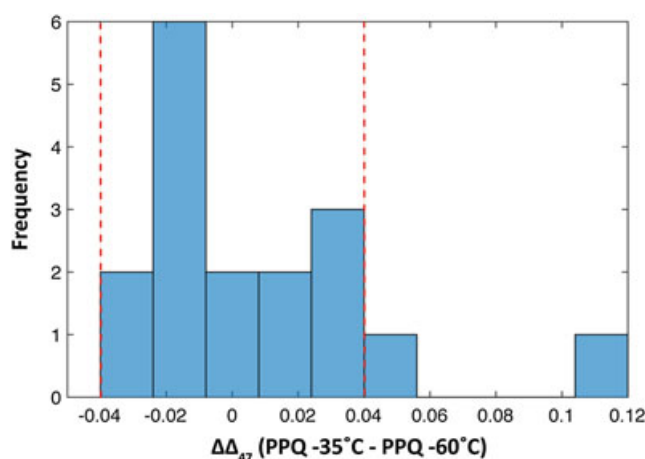


Figure 3.9 Histogram of the difference in Δ_{47} CDES between 17 samples purified at PPQ temperatures of -35°C and -60°C . Dashed red lines indicate 95% confidence level of the measurement based on Carrara marble standards

4) DISCUSSION

4.1 Impacts of contamination on Δ_{47} values

The presence of contaminant molecules within sample-derived CO_2 is believed to be a causative factor in poor reproducibility of Δ_{47} measurements. Poor mass resolution however limits our ability to identify contaminant molecules (Huntington et al., 2009).

The addition of gypsum to Carrara Marble standards has enabled us to determine the effect of increasing contaminant richness on Δ_{47} . Results indicate that whilst the addition of gypsum increases mean Δ_{47} , contaminant concentration affects Δ_{47} in a non-linear way. Whilst exposure of analyte CO_2 to the silver wool may be a significant contributing factor in the removal of sulfur compounds present, conditions of the silver trap were maintained constant

during analyses, thus its effect does not explain the lack of proportionality between weight percent gypsum added and Δ_{47} . PPQ trap temperature in these experiments was kept constant thus reducing the potential for fractionation affecting Δ_{47} measurements. We therefore propose results observed are as a consequence of volatilization of sulfur compounds and/or elemental sulfur and organic matter during acid digestion in a non-proportional relationship to overall contaminant concentration thus affecting sample reproducibility.

The lack of correlation between Δ_{48} offset and Δ_{47} in dolomite (DDOL) samples and DCM2 differs from the highly correlated relationships between small mass 47 excesses and proportionally greater mass 48 and 49 excesses observed on the addition of pentane and or CaCl_2 to whole air samples (Eiler and Schauble, 2004). Natural carbonate samples contain a greater variability of contaminant molecules than artificially spiked gases. DCM1 and modern shell samples (Figure 3.3c,e) however show good correlation between Δ_{48} offset and Δ_{47} indicating that for some materials Δ_{48} offset is a good indicator of the presence of contaminants causing interference on mass 47. The lack of correlation between Δ_{48} offset and Δ_{47} for samples DDOL and DCM2 may be attributed to contaminants such as $^{32}\text{S}^{16}\text{O}^-$, that have not been removed by exposure of analyte CO_2 to silver wool, causing interference on masses 48 and potentially 49 but not 47. The reduction of “false positives” such as these is an important factor in aiding our ability to identify contaminated samples in analyses.

The 49 parameter as defined in John and Bowen (2016) is not a widely discussed and reported metric of contamination. This is due in part to the strong correlation between mass 49 signals and pressure imbalance between the sample and reference bellows in the mass spectrometer (Bernasconi et al. 2013). Our results however illustrate that higher Δ_{47} values often correlate well with higher 49 parameter values but not necessarily with changes in Δ_{48} offset. This is especially the case for samples that exhibit “false positive” signals in Δ_{48} offset. In cases such as these the 49 parameter may prove a useful additional tool in identifying sample contamination.

The relationship between mass 47 excesses and 49 parameter is however sample dependant. In modern shell samples, DCM1 and DCM2 49 parameter values up to 0.4 produce Δ_{47} measurements within error of expected carbonate formation temperature based on Kluge et al. (2015). This differs from dolomite samples measured in which 49 parameter values are associated with proportionally much greater increases in Δ_{47} values. Consequently there is a

need to strike a balance in the choice of a threshold Δ_{49} parameter value. Selection a lower value of 0.2 may result in some sample replicates with acceptable Δ_{47} values being discarded. The choice of a higher value such as 0.4 however may result in the inclusion of sample replicates with erroneously high Δ_{47} measurements for a few materials. We take a conservative approach in maintaining use of the lower value of 0.2 in analysis.

4.2 Effect of PPQ trap temperature on $\Delta_{48\text{offset}}$ Δ_{49} parameter and Δ_{47} values

Decreasing temperature for gas chromatographic applications of some permanent gases results in an increase in the separation factor between contaminant gases and CO_2 (Szulczewski and Higuchi, 1957). Our application is in principle similar to those of gas chromatography. By lowering the temperature at which the PPQ trap is held we hoped to enhance separation between CO_2 and contaminants with longer retention times on the stable phase Porapak Q, such as sulfide gases, halocarbons and hydrocarbons thus achieving efficient CO_2 purification. This principle was tested through the purification of DDOL1.

All other laboratories using static PPQ trap for CO_2 purification are reported to operate at trap temperatures above -30°C (Petersen et al., 2015). The number of natural carbonate samples prepared at Imperial College with Δ_{48} offset values $>1.5\text{‰}$ over the period 18 February 2014 to 19 January 2015 indicate that current purification procedures may not always be sufficient to remove contamination or reduce “false positives” for sample contamination in very organic and/or sulfur-rich carbonates. We have shown that by lowering the temperature of static PPQ to -60°C during manual cryogenic carbon dioxide purification from -35°C to -60°C we achieve decreased Δ_{48} offset of sample gas from nominally pure heated regression gas lines. This increases our confidence in measured Δ_{47} organic and/or sulfur rich samples without the need for pre treatment with H_2O_2 , NaOCl or other methods shown to impact $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ values of carbonate (Lebeau et al., 2014).

The result of decreasing PPQ trap temperature is to decrease Δ_{49} parameter values in both sample types measured. This is more notable in the sample DCM2 in which decreases in Δ_{48} offset and Δ_{49} parameter are accompanied by lower sample Δ_{47} . We propose that in the case of DCM1, by lowering PPQ trap temperature we achieve improved contaminant reduction in analyte CO_2 , thus improving the reliability of our Δ_{47} measurement.

4.3 Effect of PPQ trap temperature on $\delta^{18}\text{O}$, $\delta^{13}\text{C}$ and Δ_{47} values

In the purification of carbon dioxide for isotopic analysis we strive to avoid significant isotopic fractionation, affecting either bulk or clumped isotopic value of sample gas. Isotopic values of sample DDOL1 indicate isotopic fractionation associated with the interaction of carbon dioxide with the stationary phase PPQ occurs below -45°C , leading to lower $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ values (Figure 3.6). $\Delta_{47\text{CDES}}$ of values DDOL1 purified between -25°C and -65°C however appear unaffected by PPQ temperature (Figure 3.4). The variation in $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ between at all PPQ temperatures, most likely as a result of sample contamination or inhomogeneity, make DDOL1 unsuitable to further test changes in isotopic values on lowering trap temperature.

To further test the effects of lowering PPQ trap temperature on $\delta^{18}\text{O}$, $\delta^{13}\text{C}$ and Δ_{47} values, CM and ETH3 standards were therefore purified at PPQ trap temperatures of -35°C and -60°C (Figure 3.7). Whilst isotopic fractionation at -65°C results in lower $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ values, $\Delta_{47\text{Corr}}$ appears largely unaffected by lower trap temperature. The lack of change in $\Delta_{47\text{Corr}}$ is expected, as CM and ETH3 standards are considered relatively free from contaminants, exhibiting low Δ_{48} offset and 49 parameter values when purified at -35°C . It however indicates that improvements in Δ_{47} observed in contaminated samples are most likely as a result of improved cleaning as opposed to significant fractionation in Δ_{47} .

Approximately half of samples with high $\Delta_{48\text{offset}}$ and/or 49 parameter values when purified at a PPQ trap temperature of -35°C have lower Δ_{47} values when purified at -60°C (Figure 3.9). Taking A 95% confidence level of 0.04‰ into consideration, we notice that for at least two samples the difference in Δ_{47} between the two measurements falling outside the 95% confidence interval is indicative of a statistically significant change. Thus, at least for some samples setting up the PPQ trap temperature at -60°C results in improved cleaning. Even though some samples do not exhibit lower Δ_{47} at a PPQ trap temperature of -60°C , those samples would appear contaminated at -35°C as a result of false positives for contamination on masses 48, and thus lowering of PPQ trap temperature reduces the occurrence of false positives and improves confidence in sample interpretation (Figure 3.5).

Standard operating procedure in Imperial College London is to transfer sample gas across the PPQ trap until a pressure of $< 2 \times 10^{-2}$ MPa is read on pressure gauge 1. This helps to prevent fractionation of sample gas, and is preferred to a fixed transfer time, as transfer time is dependant on both gas volume and PPQ trap temperature. Transfer times for 5 mg aliquots of

sample powder with a PPQ temperature of -35°C range from 40-50 minutes. At -60°C time taken to transfer the sample across the PPQ trap is much greater and on average 1 hour 40 minutes increasing to up to 2 hours 30 minutes for some samples. Incomplete transfer of sample gas of a portion of replicates at -60°C may result in the larger standard error in $\delta^{18}\text{O}$, $\delta^{13}\text{C}$ and $\Delta_{47\text{Raw}}$ values observed. We thus propose implementation of strict procedures with regards to transfer time and sample weight at lower Porapak-Q temperatures to ensure complete transfer of gas in all replicates. In our laboratory samples of 5 mg of 100% carbonate are transferred at -60°C for at least 1 hour 50 minutes. Whilst lowering PPQ temperature to increase the efficiency of the cleaning procedure therefore comes at the cost of longer analytical time it must be noted that the total sample preparation time from acid digestion to introduction to the mass spectrometer is still within the time taken for mass spectrometric analysis. This means that, despite the longer time taken to transfer gas across the PPQ trap, there is no change in the number of samples that can be analysed within a given time period.

4.4 Correction for fractionation in $\delta^{18}\text{O}$, $\delta^{13}\text{C}$ and $\Delta_{47\text{Raw}}$ values at lower PPQ temperatures

Our results indicate that it may not always be possible to purify at a trap temperature where all contaminants are removed (low Δ_{48}) but where $\delta^{18}\text{O}$, $\delta^{13}\text{C}$ values are unaffected by fractionation when passed through the PPQ trap. We propose correction of $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ for samples purified by manual cryogenic purification with the stable phase PPQ at -60°C through normalization to a series (at least two) of standards with differing bulk isotope ratios also purified at -60°, as implemented in the free software Easotope (John and Bowen, 2016).

This may be achieved using one of two equations:

$$\delta_{corrected} = \delta_{raw} - \frac{\sum_1^n (\delta S_{m_n} - \delta S_e)}{n} \quad (\text{Equation 3.4})$$

$$\delta_{corrected} = \delta_{raw} \times \delta_{slope} + \delta_{intercept} \quad (\text{Equation 3.5})$$

Where δ_{raw} and $\delta_{corrected}$ are sample $\delta^{13}\text{C}$ or $\delta^{18}\text{O}$ values before and after correction. In equation (4) n is the number of standards used in correction S_{m_n} and S_e are n^{th} measured δ value of the standard and the expected δ value of the standard. In equation (5) δ_{slope} and

$\delta_{intercept}$ are the slope and intercept of a linear regression through all the 'n' standards plotted in the δ_{Sm} versus δ_{Se} space (John and Bowen, 2016). This may be implemented in the free software program Easotope.

Samples with heavier bulk isotopic compositions have however been shown to have greater fractionation on lowering trap temperature than those with lighter $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ (Petersen et al., 2015). We therefore advocate the measurement of standards significantly different in $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ that bracket sample values, and recommend application of equation (5) in correction as this applies a linear regression in δ space to account for scale compression thus accounting for increased fractionation at heavier sample bulk isotopic composition. (John and Bowen, 2016) The correction of Carrara Marble and ETH3 standards purified at -60°C to within range of those purified at -30°C following this approach is demonstrated in Figure 3.10.

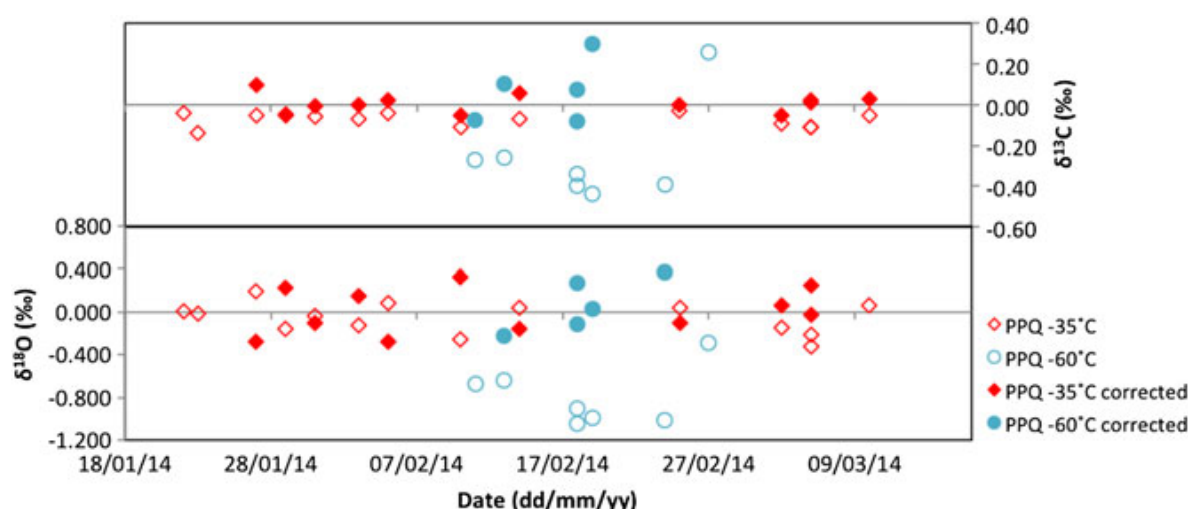


Figure 3.10 $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ corrections for Carrara marble and ETH3 Open circles and diamonds illustrate uncorrected values; filled circles and diamonds represent corrected values

In the majority of clumped isotope studies, Δ_{47} is reported in the absolute reference frame (Dennis et al. 2011) If this reference frame correction is established using standards cleaned at the same PPQ temperature as the samples, the projection in the absolute reference frame should account for fractionation in $\Delta_{47\text{Raw}}$ associated with decreased trap temperature (Petersen et al., 2015).

5) CONCLUSIONS

Our study illustrates the relationship between the metric Δ_{47} and the contamination parameters $\Delta_{48\text{offset}}$ and the 49 parameter. Δ_{47} displays a non-proportional relationship to Δ_{48} offset for some samples resulting in “false positives” for contamination. A major novel result

from this study is that reducing the Porapak-Q trap temperature to -60°C can result in improved carbon dioxide purification for contaminant-rich samples. This is manifested in a decrease in measured Δ_{48} offset values. In order to account for isotopic fractionation at -60°C samples purified at this trap temperature should be corrected using identically prepared standards. Transfer time across the Porapak trap should be closely monitored to prevent isotopic fractionation. This result has important procedural implications for any laboratories using this common technique to clean CO₂, including those using automated Kiel devices in which passive transfer across a PPQ is achieved via cryogenic trapping. Improvements in CO₂ purification allow for clumped isotopic characterization of a wider range of materials including subsurface samples found in association with hydrocarbon reserves.

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Chapter 4: The clumped (^{13}C - ^{18}O) isotope composition of echinoid calcite: further evidence for “vital effects” in the clumped isotope proxy

ABSTRACT

Carbonate clumped isotope thermometry is based on the thermodynamically dependent relative abundance of ^{13}C - ^{18}O bonds (quantified as Δ_{47}) within the carbonate crystal lattice. The clumping of ^{13}C - ^{18}O in carbonates is based on a self-reaction of isotope exchange that occurs rapidly at near neutral pH. Similar Δ_{47} -temperature relationships between biogenic and inorganically precipitated carbonate in initial studies led to the promise of a proxy free of biologically driven disequilibrium effects, commonly referred to as “vital effects”. This has been largely the case for most organisms investigated. Biologically mediated disequilibrium precipitation has however been reported in corals and cephalopods and brachiopod molluscs. Echinoderms, despite their complex inter-cellular bio-mineralization strategy, large inter-skeletal fractionation of $\delta^{18}\text{O}$, $\delta^{13}\text{C}$ and rapid calcite precipitation have however not been previously investigated with regards to their clumped isotope composition. We present clumped isotopic composition (Δ_{47}) of 25 inter-skeletal elements of 5 echinoid species with varying growth temperatures. We found no statistically significant inter-skeletal variation in Δ_{47} in all echinoid species measured, a surprising find given the important inter-skeletal variability reported for $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$. Our echinoid Δ_{47} -temperature calibration however shows a statistically significant positive offset from Δ_{47} -temperature calibration for inorganic calcite of 0.014‰. The pattern of isotopic fractionation in $\delta^{18}\text{O}$ and Δ_{47} of echinoderms is not consistent with CO_2 hydration or hydroxylation, diffusion or the high-Mg calcite composition of echinoid calcite. Positive offsets in the Δ_{47} of echinoid calcite may however relate to deviations in the pH of the calcifying fluid from the pH at which equilibrium calcite is precipitated.

1) INTRODUCTION

Measurement of the ratio of stable oxygen isotopes $^{16}\text{O}/^{18}\text{O}$ in biogenic carbonate is a well-established proxy for quantitative paleoclimate reconstruction (Urey, 1947; Epstein et al., 1951; Emiliani, 1955). This technique is however based on an *a priori* assumption of the $\delta^{18}\text{O}$ of seawater, which can vary both geographically and through time. Furthermore, while the stable oxygen isotopic composition of some taxa faithfully reflects both the temperature and

the $\delta^{18}\text{O}$ of seawater, other taxa show specific discrepancies known as “vital effects” (Epstein et al., 1951).

Carbonate clumped isotope thermometry is a temperature based proxy examining the thermodynamically dependent “clumping” of ^{13}C and ^{18}O within the carbonate crystal lattice (Eiler and Schauble, 2004; Ghosh et al., 2006; Eiler, 2007). This is based on the homogeneous isotope exchange equilibrium $\text{M}^{13}\text{C}^{16}\text{O}_3 + \text{M}^{12}\text{C}^{18}\text{O}^{16}\text{O}_2 \rightleftharpoons \text{M}^{13}\text{C}^{18}\text{O}^{16}\text{O}_2 + \text{M}^{12}\text{C}^{16}\text{O}_3$ (where M is a metal such as Ca), a system that undergoes rapid oxygen isotope exchange at a near neutral pH. At low temperatures the formation of ^{13}C - ^{18}O bonds is promoted as the vibrational energy in a heavy-heavy bond is less than half that of the corresponding light –light bond. Decreasing temperature thus favors the formation of $\text{M}^{13}\text{C}^{18}\text{O}^{16}\text{O}_2$ (Schauble et al., 2006). The excess of $^{13}\text{C}^{18}\text{O}^{16}\text{O}$ relative to a stochastic distribution, in which all isotopes are equally distributed among isotopologues, is then measured and expressed using the metric Δ_{47} defined as:

$$\Delta_{47} = \left[\left(\frac{R^{47}}{R^{47*}} - 1 \right) - \left(\frac{R^{46}}{R^{46*}} - 1 \right) - \left(\frac{R^{45}}{R^{45*}} - 1 \right) \right] \times 1000$$

(Equation 4.1)

Where $R_i = \text{mi}/\text{m44}$ and R^* gives the abundance of each isotopologue of a gas with the same bulk isotopic composition as R but with a stochastic distribution (Affek and Eiler, 2006). As this is a self-reaction of isotope exchange, the estimate for the carbonate formation temperature is independent of the oxygen isotopic composition of the parent fluid (Eiler, 2007). This is particularly useful in climate reconstruction where determination of the temperature and $\delta^{18}\text{O}$ composition of water is important in discerning other environmental factors such as geographic distribution of ice, ocean circulation, and the hydrological cycle (Eiler, 2011).

The term “vital effects” refers to the offset between the isotopic compositions of biogenic carbonates and inorganic carbonates precipitated under identical conditions. The success of the clumped isotope thermometer has a strong basis in the congruence between empirical calibrations using biogenic carbonates (Ghosh et al., 2006; Ghosh et al., 2007; Came et al., 2007; Eagle et al., 2010) and calibrations based on inorganic precipitation experiments (Ghosh et al., 2006; Dennis and Schrag, 2010). In particular groups that show significant non-equilibrium fractionation of $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ appear free of vital effects in their clumped isotopic composition (Tripathi et al., 2010; Thiagarajan et al., 2011; Grauel et al., 2013; Katz et al.,

2017). Furthermore empirical calibrations are in good accordance with theoretical calculations of isotopic clumping on varying temperature (Schauble et al., 2006; Guo et al., 2009).

Subtle non-equilibrium fractionations in the clumped isotope composition of shallow warm-water corals (Saenger et al., 2012), deep-sea cold-water corals (Thiagarajan et al., 2011; Spooner et al., 2016), cephalopods (Dennis et al., 2013) and molluscs (Henkes et al., 2013) have however been reported. Deviations from equilibrium precipitation, as defined by inorganic precipitation experiments, are group dependent and tend to both higher and lower Δ_{47} (Passey and Henkes, 2012; Saenger et al., 2012; Dennis et al., 2013; Henkes et al., 2013; Kimball et al., 2015). The drivers of group specific non-equilibrium fractionations however remain elusive and are complicated by uncertainties in how well inorganic calibrations reflect true equilibrium values (Zaarur et al., 2013).

Improvements in data processing for clumped isotope analysis (Daëron et al., 2016; Schauer et al., 2016) in association with comprehensive inorganic calibration of the thermometer over a wide temperature range and steps towards the development of a “universal temperature- Δ_{47} calibration” (Kluge et al., 2015; Bonifacie et al., 2016; Kelson et al., 2017) have reduced uncertainties in the true slope and intercept of the inorganic Δ_{47} -temperature relationship. It is therefore now easier to assess non-equilibrium fraction in the clumped isotope composition of biogenic carbonate, notably in groups that show a strong disequilibrium signal in $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$.

One such group is echinoderms. The stable isotopic composition of echinoid skeletal elements has been well characterised (Weber and Raup, 1966a; Weber and Raup, 1966b; Weber, 1968; Courtney and Ries, 2015). Inter-skeletal elements differ by several parts per thousand in $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ with enrichment of $\delta^{13}\text{C}$ of up to 12‰ in test calcite in comparison with spines. $\delta^{18}\text{O}$ fractionation in echinoid spines is inversely related to temperature while $\delta^{13}\text{C}$ in spines is positively correlated with atmospheric $p\text{CO}_2$. Echinoids are capable of rapid calcification (Gorzela 2011). $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ e also correlate with calcification rate (Courtney and Ries, 2015). Correcting for the effects of growth temperature, atmospheric $p\text{CO}_2$ and calcification rate on $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ fractionation $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ remain positively correlated indicating the presence of un-accounted for vital effects (Courtney and Ries, 2015).

Echinoids have a unique strategy of intercellular biomineralization, depositing high magnesium calcite (Chave, 1954; Schroeder et al., 1969; McClintock et al., 2011) on an organic matrix to generate a three-dimensional mesh known as a stereom with an initial phase of ACC deposition (Markel et al., 1986). Demonstrated links between calcification rate and vital effects in stable $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ isotopic composition, unique biomineralization strategy and large magnitude of isotopic fractionation, especially in $\delta^{13}\text{C}$, make the echinoids an excellent group in which to test for “vital effects” in clumped isotope composition.

Here we present the first systematic measurements of the clumped isotopic composition (Δ_{47}) of modern echinoderm calcite. This study both adds to existing biogenic calibration of the clumped isotope analysis and adds to our understanding of vital effects acting upon the clumped isotope composition of calcite.

2) METHODOLOGY

2.1 Samples

Echinoid samples were collected at four different localities around the globe mostly live from the benthos with one specimen grown by aquaculture (Table 1). Live collection of specimens enables us to discount post-mortem mineralogical alteration from high-Mg calcite. It also minimises the potential for post-mortem isotopic alteration in the littoral zone, either by marine or meteoric diagenesis. Echinoid species *Diadema Setosum* and *Tripneustes Gratilla* were fished in Indonesian waters and transported live to Imperial College where they were maintained in an aquarium at 25°C. Echinoid species *Paracentrotus Lividus* were collected live at 1.5 m depth in the eastern Mediterranean. Deceased irregular echinoids *Dendraster Excentricus* were sampled from the foreshore at Morro Bay on the Pacific coast, USA. Only *Dendraster Excentricus* specimens with spines still attached and organic components still present were selected ensure selected specimens were recently deceased. *Stronglyocentrotus Droebachiensis*, hatched and grown in an aquaculture facility, were provided by the Centre for co-operative Aquaculture, University of Maine. Growth by aquaculture provides a very well constrained record of growth temperature over the lifespan of the echinoid. Echinoid samples were classified by order (Table 1) and are addressed as such in results and discussion sections for ease of referral.

Table 1

Specimen sampling details and collection location. Growth water temperature and stable oxygen isotopic composition of analysed samples

Sample Information					Seawater properties	
Sample name	Order	Genus	Inter-skeletal element	Location	Temperature (°C) ^a	$\delta^{18}\text{O}_{\text{water}}$ VSMOW (‰) ^b
GA1	<i>Temnopleuroidea</i>	<i>Tripneustes</i>	Ambulacral Column	Indonesia, Java Sea	28.4 ± 0.15	0.28 ± 0.02
GA2	<i>Temnopleuroidea</i>	<i>Tripneustes</i>	Interambulacral Column	Indonesia, Java Sea	27.8 ± 0.15	0.28 ± 0.02
GLT	<i>Temnopleuroidea</i>	<i>Tripneustes</i>	Tooth	Indonesia, Java Sea	27.8 ± 0.15	0.28 ± 0.02
GS	<i>Temnopleuroidea</i>	<i>Tripneustes</i>	Spine	Indonesia, Java Sea	27.8 ± 0.15	0.28 ± 0.02
GT	<i>Temnopleuroidea</i>	<i>Tripneustes</i>	Bulk Test	Indonesia, Java Sea	27.8 ± 0.15	0.28 ± 0.02
BS	<i>Diadematoidea</i>	<i>Diadema</i> sp.	Spine	Indonesia, Java Sea	27.8 ± 0.15	0.28 ± 0.02
BT	<i>Diadematoidea</i>	<i>Diadema</i> sp.	Bulk Test	Indonesia, Java Sea	27.8 ± 0.15	0.28 ± 0.02
MA1	<i>Clypeasteroidea</i>	<i>Dendraster</i>	Ambulacral Column	Morro Bay, CA	13.4 ± 0.03	-0.57 ± 0.03
MA2	<i>Clypeasteroidea</i>	<i>Dendraster</i>	Interambulacral Column	Morro Bay, CA	13.4 ± 0.03	-0.57 ± 0.03
MB	<i>Clypeasteroidea</i>	<i>Dendraster</i>	Base of test	Morro Bay, CA	13.4 ± 0.03	-0.57 ± 0.03
ML	<i>Clypeasteroidea</i>	<i>Dendraster</i>	Lantern (teeth, pyramid, rotula)	Morro Bay, CA	13.4 ± 0.03	-0.57 ± 0.03
MP	<i>Clypeasteroidea</i>	<i>Dendraster</i>	Petal	Morro Bay, CA	13.4 ± 0.03	-0.57 ± 0.03
MS	<i>Clypeasteroidea</i>	<i>Dendraster</i>	Spine	Morro Bay, CA	13.4 ± 0.03	-0.57 ± 0.03
YA1	<i>Camarodonta</i>	<i>Paracentrotus</i>	Ambulacral Column	San Andrea, Elba, IT	18.5 ± 0.54	1.36 ± 0.03
YA2	<i>Camarodonta</i>	<i>Paracentrotus</i>	Interambulacral Column	San Andrea, Elba, IT	18.5 ± 0.54	1.36 ± 0.03
YLP	<i>Camarodonta</i>	<i>Paracentrotus</i>	Pyramid	San Andrea, Elba, IT	18.5 ± 0.54	1.36 ± 0.03
YLR	<i>Camarodonta</i>	<i>Paracentrotus</i>	Rotula	San Andrea, Elba, IT	18.5 ± 0.54	1.36 ± 0.03
YLT	<i>Camarodonta</i>	<i>Paracentrotus</i>	Teeth	San Andrea, Elba, IT	18.5 ± 0.54	1.36 ± 0.03
YS	<i>Camarodonta</i>	<i>Paracentrotus</i>	Spine	San Andrea, Elba, IT	18.5 ± 0.54	1.36 ± 0.03
YT	<i>Camarodonta</i>	<i>Paracentrotus</i>	Bulk Test	San Andrea, Elba, IT	18.5 ± 0.54	1.36 ± 0.03
SDA1	<i>Strongylocentrotus</i>	<i>Droebachiensis</i>	Ambulacral Column	University of Maine	8.5 ± 0.2	-1.2 ± 0.009
SDA2	<i>Strongylocentrotus</i>	<i>Droebachiensis</i>	Interambulacral Column	University of Maine	8.5 ± 0.2	-1.2 ± 0.009
SDS	<i>Strongylocentrotus</i>	<i>Droebachiensis</i>	Spine	University of Maine	8.5 ± 0.2	-1.2 ± 0.009
SDLT	<i>Strongylocentrotus</i>	<i>Droebachiensis</i>	Tooth	University of Maine	8.5 ± 0.2	-1.2 ± 0.009
SDT	<i>Strongylocentrotus</i>	<i>Droebachiensis</i>	Bulk Test	University of Maine	8.5 ± 0.2	-1.2 ± 0.009

^a Ocean temperature estimated from the NOAA World Ocean Atlas 2013 (v.2) (Locarnini *et al.* 2013). Standard error reflects difference in seasonal temperatures over the habitual depth range of groups listed ^b Water $\delta^{18}\text{O}$ values estimated from the global gridded data set of oxygen isotope composition in water (LeGrande & Schmidt 2006) excluding *Strongylocentrotus Droebachiensis* where water $\delta^{18}\text{O}$ was directly measured from an aliquot taken from the aquaculture tank

Table 2

Sample stable and clumped isotope composition

Sample name	Order	Genus	n	$\delta^{18}\text{O}$ VPDB		$\delta^{13}\text{C}$ VPDB		$\Delta_{47}\text{CDES}$ (‰)	S.E
				(‰)	S.E	(‰)	S.E		
GA1	<i>Temnopleuroidea</i>	<i>Tripneustes</i>	3	-1.59	0.04	1.16	0.02	0.710	0.006
GA2	<i>Temnopleuroidea</i>	<i>Tripneustes</i>	3	-1.39	0.05	1.20	0.02	0.703	0.005
GLT	<i>Temnopleuroidea</i>	<i>Tripneustes</i>	5	-1.69	0.07	0.67	0.02	0.713	0.011
GS	<i>Diadematoidea</i>	<i>Diadema sp.</i>	3	-1.64	0.18	0.28	0.01	0.696	0.012
GT	<i>Diadematoidea</i>	<i>Diadema sp.</i>	3	-1.32	0.17	1.25	0.06	0.710	0.012
BS	<i>Temnopleuroidea</i>	<i>Tripneustes</i>	3	-1.39	0.06	0.01	0.06	0.713	0.005
BT	<i>Temnopleuroidea</i>	<i>Tripneustes</i>	3	-1.75	0.10	-2.03	0.02	0.727	0.004
MA1	<i>Clypeasteroidea</i>	<i>Dendraster</i>	3	1.41	0.09	-0.19	0.05	0.771	0.005
MA2	<i>Clypeasteroidea</i>	<i>Dendraster</i>	3	1.49	0.10	-0.03	0.02	0.760	0.004
MB	<i>Clypeasteroidea</i>	<i>Dendraster</i>	3	1.29	0.14	-0.16	0.01	0.743	0.005
ML	<i>Clypeasteroidea</i>	<i>Dendraster</i>	3	1.12	0.15	-1.35	0.01	0.756	0.010
MP	<i>Clypeasteroidea</i>	<i>Dendraster</i>	3	1.18	0.03	-0.69	0.05	0.763	0.012
MS	<i>Clypeasteroidea</i>	<i>Dendraster</i>	3	1.03	0.08	-0.55	0.01	0.746	0.006
YA1	<i>Camarodonta</i>	<i>Paracentrotus</i>	3	0.86	0.13	-1.22	0.01	0.711	0.012
YA2	<i>Camarodonta</i>	<i>Paracentrotus</i>	4	0.69	0.07	-1.48	0.05	0.697	0.015
YLP	<i>Camarodonta</i>	<i>Paracentrotus</i>	3	1.33	0.05	-1.51	0.08	0.732	0.015
YLR	<i>Camarodonta</i>	<i>Paracentrotus</i>	3	1.00	0.05	-2.22	0.01	0.726	0.006
YLT	<i>Camarodonta</i>	<i>Paracentrotus</i>	3	0.85	0.12	-0.93	0.03	0.721	0.011
YS	<i>Camarodonta</i>	<i>Paracentrotus</i>	3	1.79	0.21	0.81	0.03	0.726	0.010
YT	<i>Camarodonta</i>	<i>Paracentrotus</i>	3	0.60	0.11	-1.64	0.02	0.735	0.005
SDA1	<i>Strongylocentrotus</i>	<i>Droebachiensis</i>	3	-0.30	0.05	-3.23	0.02	0.784	0.007
SDS	<i>Strongylocentrotus</i>	<i>Droebachiensis</i>	3	0.07	0.09	-1.32	0.01	0.755	0.002
SDA2	<i>Strongylocentrotus</i>	<i>Droebachiensis</i>	3	-0.24	0.02	-3.02	0.04	0.782	0.007
SDLT	<i>Strongylocentrotus</i>	<i>Droebachiensis</i>	3	0.03	0.10	-2.40	0.02	0.767	0.009
SDT	<i>Strongylocentrotus</i>	<i>Droebachiensis</i>	4	-0.51	0.11	-3.46	0.03	0.766	0.009

n is number of replicates

Growth temperatures of samples collected from the benthos were estimated from the World Ocean Database (V.2) (Locarnini et al. 2013). over estimated specimen growth depth. This depth was 0-10m for *Diadema Setosum* and *Tripneustes Gratilla* (Cleary et al., 2005; Ristanto et al., 2018) 0-5m for *Dendraster Excentricus* (Merill and Hobson, 1970) and 0-5m as sampled for *Paracentrotus Lividus*. Average and standard deviation in annual seawater temperature at each collection location is taken from the World Ocean Database (V2) (Locarnini et al. 2013). The growth temperature of *Stronglyocentrotus Droebachiensis* is calculated from weekly temperature measurements within the aquarium over the course of specimen growth.

Seawater $\delta^{18}\text{O}$ values were estimated from the global gridded dataset of LeGrande and Schmidt (2006). Where possible values were assumed over the depth range of the species, otherwise sea surface measurements (0m depth) are used. Database $\delta^{18}\text{O}_{\text{sw}}$ is converted into expected $\delta^{18}\text{O}_{\text{carbonate}}$ using the calibration of (Mavromatis et al., 2012). Expected $\delta^{18}\text{O}_{\text{carbonate}}$ VSMOW (‰) is then converted into $\delta^{18}\text{O}_{\text{carbonate}}$ VPDB (‰) using established equation $\delta^{18}\text{O}_{\text{VPDB}} = 0.97001, \delta^{18}\text{O}_{\text{VMOW}} - 29.99$ (Coplen, 1988).

2.2 Stable isotopic measurements

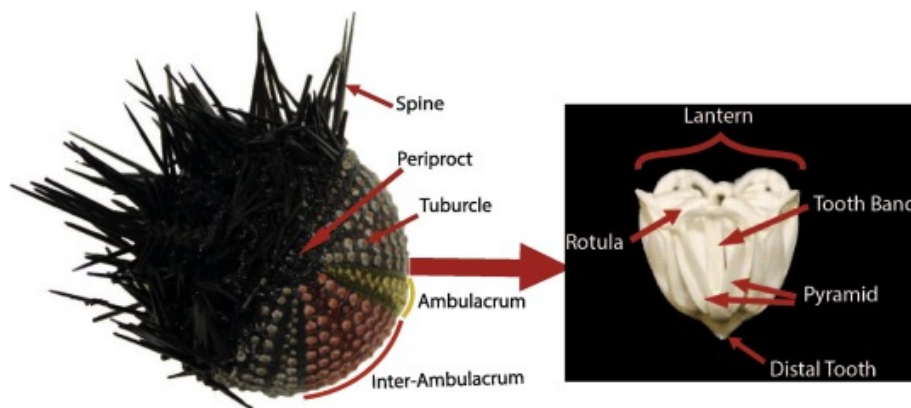


Figure 4.1 Illustration of inter-skeletal elements sampled within echinoids. Echinoid pictured is *Paracentrotus Lividus*

We sampled echinoid inter-skeletal elements as illustrated in Figure. 4.1. Ambulacral regions such as those shaded red and interambulacral regions such as those shaded in yellow (Figure. 4.1) were powdered using a dental drill. Elements of the lantern were disarticulated, separated and powdered using a pestle and mortar. The longest spines were selected from the region of largest test diameter, removed and gently crushed with a pestle and mortar. We also took “bulk” test measurements consisting of over

3cm² areas of test calcite composing both ambulacral and interambulacral regions which were separately crushed.

Isotopic analyses were carried out in the Qatar Stable Isotope Laboratory at Imperial College London. Typical sample sizes were in the range of 5-8 mg per replicate.

To remove organic contaminants all samples were treated by low temperature plasma ashing under oxygen at a flow rate of 400 ml/min power of 1000 w with a treatment time of between 30 and 45 minutes. Lebeau et al (2014) recommend low temperature ashing for measurement of stable $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ as it facilitates the removal of organic matter whilst preserving isotopic abundances. In order to ensure low temperature oxygen plasma ashing has not affected Δ_{47} of echinoid samples we treated carbonate standard ETH3 using the same technique. Δ_{47} of plasma treated ETH3 standard ($\mu = 0.71 \pm 0.015$, $n = 8$) is within range of untreated ETH3 standard measured at Imperial over the same measurement period ($\mu = 0.69 \pm 0.015$, $n = 8$). We can thus assume that positive offset in echinoid Δ_{47} from the inorganic calibration is not as a result of difference in analytical protocol. We see no significant effect on stable $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ of ETH3 on oxygen plasma ashing these being $\delta^{18}\text{O}_{\text{VPDB}} = -1.74$, $\delta^{13}\text{C}_{\text{VPDB}} = 1.70$ and being $\delta^{18}\text{O}_{\text{VPDB}} = -1.76$, $\delta^{13}\text{C}_{\text{VPDB}} = 1.69$ in non-treated and treated samples respectively.

The relative abundance of the $^{13}\text{C}^{18}\text{O}^{16}\text{O}$ isotopologue in acid evolved carbon dioxide is directly proportional to the extent of ^{13}C - ^{18}O ordering in the carbonate phase (Ghosh et al., 2006). Powdered samples are thus reacted individually in 105% orthophosphoric acid at 90°C for 10 minutes following (McCrea, 1950) and (Swart et al., 1991). Evolved carbon dioxide was purified using a series of water traps and “static” Porapak -Q packed U-trap through which gas is transferred without helium flow using both the pull of liquid nitrogen and a pressure differential across the trap (Davies and John, 2017; **chapter 2**).

Analyte CO_2 was measured on dual inlet Thermo 253 mass spectrometer with faraday cups registered through m/z 44-49. The dual inlet system allows rapid repeated inter-comparison between sample and reference gas, a CO_2 standard from Oztech $\delta^{13}\text{C}_{\text{VPDB}} - 3.64\text{‰}$, $\delta^{18}\text{O}_{\text{VSMOW}} 25.01\text{‰}$. Measurements comprised 8 acquisitions each with 7

cycles with 26 s integration time. A typical acquisition time is 20 minutes corresponding to a total analysis time of 2.5-3 h. This measurement is repeated at least three times per sample.

2.3 Data analysis and reduction

The $^{13}\text{C}^{18}\text{O}^{16}\text{O}$ isotopologue constitutes approximately 97% of the abundance of CO_2 measured on mass 47 (Eiler, 2007). The isotopic parameters of (Brand et al., 2010) are used in calculation of raw Δ_{47} , as these have been shown to better account for anomalies in sample ^{17}O thus produce accurate Δ_{47} values independent of sample bulk isotopic composition (Daëron et al., 2016; Schauer et al., 2016). All calculations and corrections are carried out in the freely available stable isotope management software *Easotope* (John and Bowen, 2016).

Raw Δ_{47} is corrected in three steps. In order to correct for non-linearity of the mass spectrometer, heated gasses with different bulk isotopic compositions, were measured and normalized by projecting all Δ_{47} measurements to a δ_{47} of 0. Heated gasses were prepared following the procedure described in **chapter 2**. Δ_{47} measurements corrected for non-linearity were then translated into the absolute reference frame of (Dennis et al., 2011). Carbonate standards routinely measured to allow for translation into the absolute reference frame are Carrara Marble, ETH2 and ETH3. Full details of the heated gas and carbonate standards run over the course of the measurement period can be found in the supplementary material (Annex 1). Δ_{47} is corrected for acid fractionation by adding the acid fractionation factor 0.082‰ (Defliese et al., 2015). $\delta^{18}\text{O}$ is corrected for phosphoric acid digestion at 90°C using an acid fractionation factor of 1.0081‰ (Kim et al., 2007)

Masses 48 and 49 were used to detect the presence of contaminants. Samples with Δ_{48} offset values >1.5‰ or 49 parameter values >0.2 were excluded from analysis following Davies and John (2017).

2.4 Statistical Methods

Statistical analysis was carried out using the software *JMP® version 13.2.0*. The appropriate application of statistical methods strengthens conclusions based on available data. The Δ_{47} , $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ values of homogenous carbonate standards (e.g.

Carrara Marble $n = 53$, ETH3 $n = 147$) are normally distributed. Therefore, we assume normality in the distribution of our sample data ($n = 3-4$), allowing for the application of parametric statistical tests. Average standard deviation (σ) sample on Δ_{47} for 3-4 replicates is 0.0147‰.

One-way ANOVA (ANalysis Of VAriance) tests enable us to determine if data of sample size N from a given number groups k of equal or unequal size n have a common mean m . The null hypothesis assumes a common mean. It is a parametric test but robust to modest violations in sample normality. One way-ANOVA tests are reported following APA style (Association, 1994). If the null hypothesis is rejected by one-way ANOVA we carry post-hoc pairwise student t-tests to confirm where difference occurs between groups. We then use effect size in association with group number, sample size and significance level to calculate statistical power using G*Power (Faul et al., 2007). Power values tells us the probability of detecting a “true” effect i.e. rejecting a false null hypothesis. Low power values indicate a smaller chance of rejecting a false null hypothesis. Further details of these calculations are included in Appendix 1.

ANCOVA (ANalysis Of COVAriance) tests are used to determine differences in Δ_{47} -temperature relationship between inorganic and biogenic calibrations. ANCOVA combines ANOVA and regression analysis enabling the user to determine if groups have a common mean m , whilst controlling for the effect of covariates. As with ANOVA we carry out carry post-hoc pairwise student t-tests to confirm where difference occurs between groups and use effect size in combination with sample size to calculate statistical power at a significance level of 0.05 (Table 4).

3) RESULTS

The isotopic compositions ($\delta^{13}\text{C}$, $\delta^{18}\text{O}$ and Δ_{47}) of inter-skeletal elements of all echinoids are shown in Table 2.

3.1 Variation in $\delta^{13}\text{C}$, $\delta^{18}\text{O}$ in echinoid calcite

The $\delta^{18}\text{O}_{\text{PDB}}$ of distinct skeletal elements in each specimen vary (Figure 4.2). One-way ANOVA tests show that differences in the $\delta^{18}\text{O}_{\text{PDB}}$ of inter-skeletal elements in echinoid

orders *Diadematoidea*, *Camarodonta* and *Strongylocentrotus* are statistically significant at the 95% confidence level (Table 3).

Table 3

Summary of ANOVA statistics for the effect of inter-skeletal element on $\delta^{18}\text{O}$ and Δ_{47} for echinoid groups measured

ANOVA				
Inter-skeletal Element vs. $\delta^{18}\text{O}$	DF(Groups)	DF(Error)	F-value	p-value
<i>Diadema</i> sp.	1	4	9.2783	0.0382
<i>Tripnesutes</i>	4	12	2.303	0.1182
<i>Dendraster</i>	5	12	2.6687	0.0759
<i>Paracentrotus</i>	6	15	12.8547	<0.001
<i>Droebachiensis</i>	4	11	7.6471	0.0034
Inter-skeletal Element vs. Δ_{47}				
<i>Diadema</i> sp.	1	4	5.0797	0.0873
<i>Tripneustes</i>	4	12	0.4471	0.7727
<i>Dendraster</i>	5	12	2.1503	0.1287
<i>Paracentrotus</i>	6	15	1.4311	0.2669
<i>Droebachiensis</i>	4	11	2.3054	0.1232

Individuals from echinoid orders *Camarodonta*, *Strongylocentrotus* and *Clypeasteroidea* exhibit linearly correlated inter skeletal $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ (Figure 4.2). These groups also show

an overall trend of linearly correlated $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ between different individuals ($R^2 = 0.785$, $P < 0.001$). Urchin orders *Temnopleuroidea* and *Diadematoidea*, collected in the Indonesian region at similar growth temperatures, show only small inter-skeletal variations in $\delta^{18}\text{O}$ (0.19‰ and 0.37‰ respectively), which are poorly correlated with differences in $\delta^{13}\text{C}$.

Inter-skeletal variation in $\delta^{13}\text{C}$ is greater than that for $\delta^{18}\text{O}$, individual skeletal elements exhibiting distinct $\delta^{13}\text{C}$ signatures (Figure 4.2). In measured specimen of the order *Temnopleuroidea* calcite sampled from spines has the lowest $\delta^{13}\text{C}$ of 0.41‰ while test calcite has a $\delta^{13}\text{C}$ of 1.35‰. Conversely in the measured specimen of the order *Camarodonta*, calcite with the highest $\delta^{13}\text{C}$ is found in the spines (0.81‰) while test and lantern calcite is much lighter in $\delta^{13}\text{C}$ (-0.93 to -2.22‰).

The natural logarithm of the oxygen isotope fractionation factor ($\ln 1000\alpha_{\text{calcite-H}_2\text{O}}$) between echinoid calcite and seawater is positively correlated ($R^2 = 0.857$, $P < 0.001$), with a least-squared regression best fit equation of $\ln 1000\alpha_{\text{calcite-H}_2\text{O}} = 14.70 \times$

$(1000/T) - 20.13$, where 'T' is temperature in °K. Although oxygen isotope fractionation is correlated with temperature, $\delta^{18}\text{O}$ values of echinoid calcite are offset from temperature dependent equilibrium as established by inorganic precipitation experiments (Mavromatis et al., 2012) (Figure 4.3). Four of five echinoid groups measured exhibit positive offsets in $\delta^{18}\text{O}$ in comparison with inorganic $\delta^{18}\text{O}$ calibration. The value of the offset varies between 0.65-1.25‰ in measured specimens of orders *Temnopleuroida* and *Diadematoidea* to >3.7‰ in *Clypeasteroidea*. The measured specimen of order *Strongylocentrotus* however exhibits a negative offset in $\delta^{18}\text{O}$ from the inorganic calibration of up to 1.34‰. As importantly, the slope of the $\ln 1000\alpha_{\text{calcite-H}_2\text{O}}$ in echinoids differs from that of inorganic precipitation experiments (Mavromatis et al., 2012) (Figure 4.3).

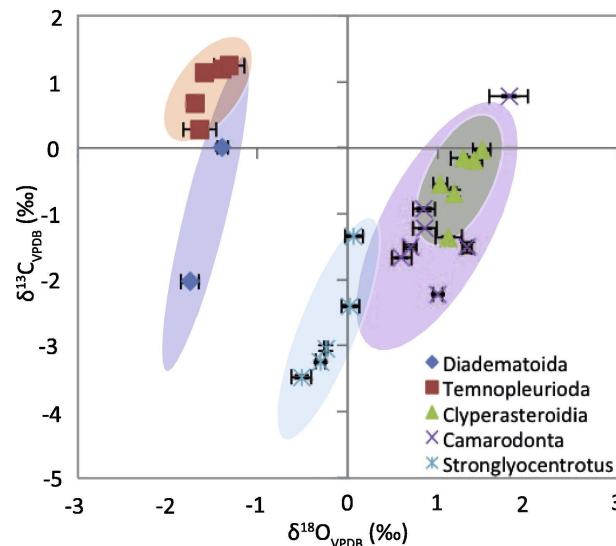


Figure 4.2 Correlation between stable $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ in echinoid calcite. 90% confidence ellipses for each echinoid order are shown as shaded areas

3.2 Inter-skeletal Δ_{47}

Individual Δ_{47} measurements of inter-skeletal elements lie within 95% confidence intervals for all echinoid species, most falling within 1σ (on average 0.0147‰, Figure 4.4). One-way ANOVA tests show there is no statistically significant difference in Δ_{47} values of echinoid inter-skeletal elements at the 95% confidence level (Table 3). This is however associated with a low power value indicating low likelihood of rejecting the null hypothesis as a result of sample size. There is correspondingly no correlation between inter-skeletal $\delta^{18}\text{O}$ and Δ_{47} (Figure 4.4) or $\delta^{13}\text{C}$ and Δ_{47} .

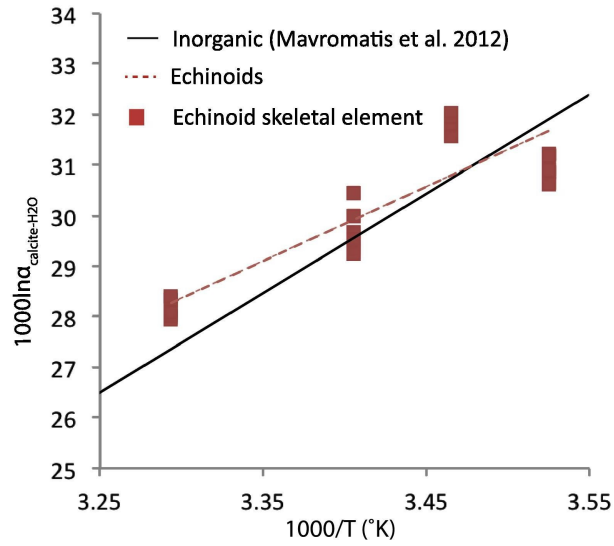


Figure 4.3 Comparison of temperature dependant fractionation between calcite and water in echinoids and inorganic precipitation experiments of Mavromatis et al. (2012)

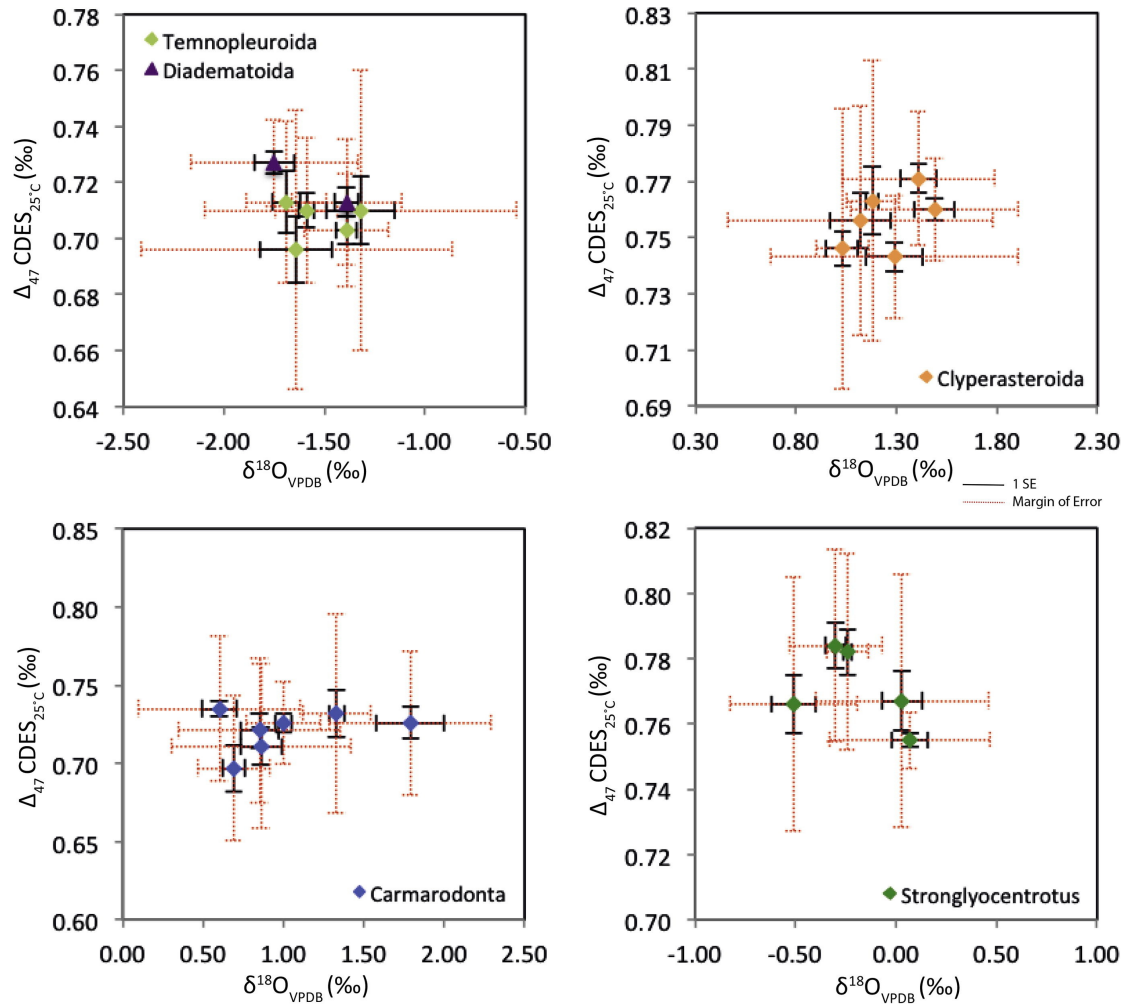


Figure 4.4 Correlation between echinoid $\delta^{18}\text{O}$ and Δ_{47} for different echinoid groups. Black error bars indicate 1 SE red error bars reflect margin of error at the 95% CI. Note that Δ_{47} CDES is presented at an acid digestion temperature of 25°C by adding a correction factor of 0.082‰ (Defliese et al. 2015).

3.3 Echinoid Δ_{47} –temperature relationship

Mean Δ_{47} values of 24 inter-skeletal measurements of 5 echinoid groups are plotted versus mean annual growth temperatures (Figure 4.5). A linear least squares regression of this data yields the following relationship:

$$\Delta_{47} = 0.03922 \times \frac{10^6}{T^2} + 0.2653 \quad (\text{Equation 4.2})$$

($R^2 = 0.62$, $P < 0.001$) where T is temperature in °K.

Table 4

Summary of ANCOVA and student t-test statistics for variation in $\delta^{18}\text{O}$ and Δ_{47} for all echinoid groups measured

	Ancova		Student t-test					
	DF (Groups)	DF (Error)	F-value	p-value	α	t	Effect size (d)	Power
Effect of inorganic calibration, Kluge et al. (2015) vs Kelson et al. (2017)	1	81	2.451	0.1214	0.05	1.98969	0.020200463	0.0505
Vital effects in Δ_{47} -T calibration, echinoids v.s inorganic calibration	1	108	15.39	0.002	0.05	1.9826	0.4251	0.3135

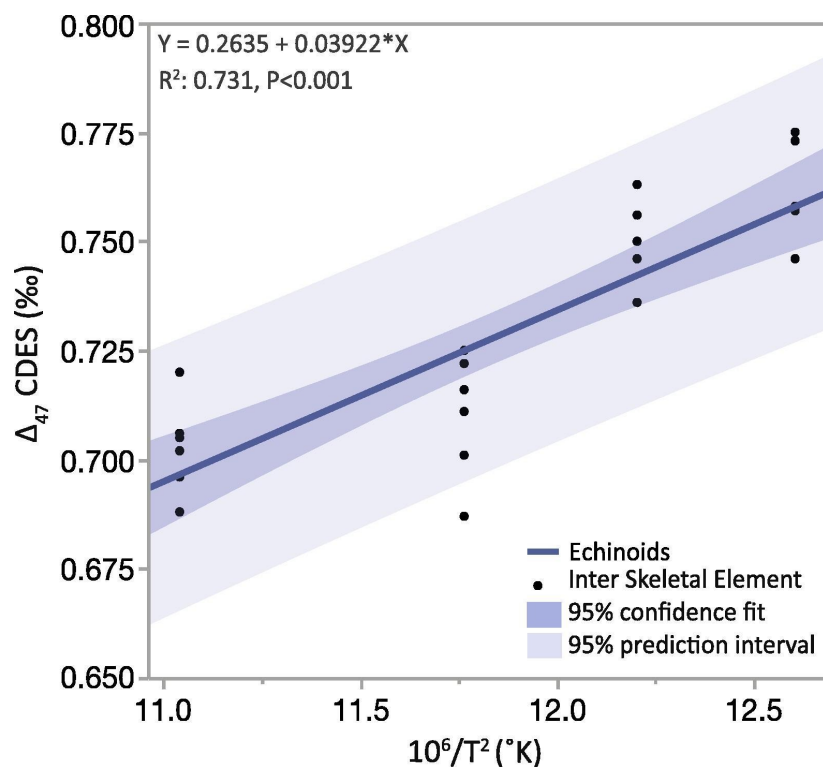


Figure 4.5 Correlation between temperature and Δ_{47} in echinoids. Black data circles represent clumped isotope measurements of inter-skeletal elements of each echinoid order comprising 3-4 replicates

4) DISCUSSION

4.1 Echinoid Δ_{47} vs. combined inorganic calibration

We compare the echinoid T- Δ_{47} with expected values based on the inorganic Δ_{47} -temperature calibration experiments. We chose to compare the calibrations of Kluge et al., (2015) and Kelson et al. (2017) with our echinoid dataset. These inorganic calibrations have been selected as they encompass multiple precipitation methods, cover a wide temperature range and incorporate a high density of measurements in the low temperature range (Figure 4.6).

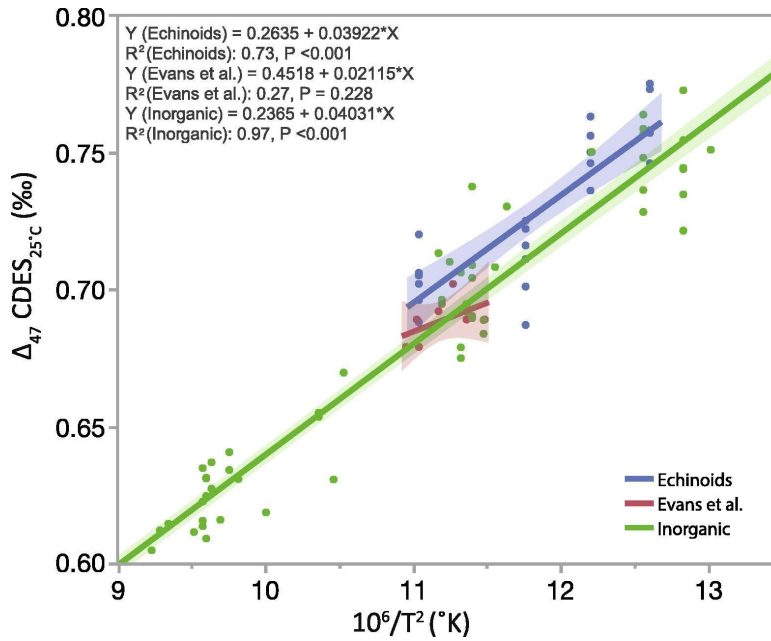


Figure 4.6 Correlation between temperature and Δ_{47} in echinoids, the combined inorganic calibration of Kluge et al. 2015 and Kelson et. al. 2017 and Evans et al. (2018). Δ_{47} CDES is presented at 25°C acidification temperature by adding the acid fractionation factor 0.082 (Defliese et al. 2015). Note the negligible overlap in confidence fits between the echinoid inorganic calibrations and 0.015% positive offset of the echinoid calibration

In order to allow direct comparison with other calibrations and with our own study, the Kluge et al. (2015) dataset has been re-processed using the Brand et al. (2010). ^{17}O correction parameters, and presented in the $\Delta_{47}\text{CDES}_{25^\circ\text{C}}$ reference frame using the acid fractionation factor of Defliese et al. (2015) (Annex 2) . This yields the following equation:

$$\Delta_{47\text{CDES}_{25^\circ\text{C}}} = 0.03998 \times \frac{10^6}{T^2} + 0.2423 \quad (\text{Equation 4.3})$$

where T is temperature in °K. The Kluge et al. (2015) calibration re-processed using the parameters of Brandt et al. (2010) is particularly useful as it was generated at Imperial College London following almost identical CO_2 purification and IRMS analyses as our echinoids measured.

An ANCOVA test conducted indicates there is significant difference between the inorganic calibrations of Kluge et al. (2015) and Kelson et al. (2017) ($F(1,81) = 2.4510$, $P = 0.1214$) correcting for temperature. Kluge *et al.* 2015 and Kelson *et al.* 2017 have no

statistical difference in slope ($F(1,81) = 1.2953$, $P = 0.2585$) A student's t-test of difference in regression by least squares means between ($\alpha = 0.05$, $t = 1.98969$) verifies that there is no significant difference in intercept between the two calibrations (Table 4). Kluge et al. (2015) and Kelson et al. (2017) are thus combined to form a general inorganic calibration with equation

$$\Delta_{47_{\text{CDES}_{25^{\circ}\text{C}}}} = 0.04031 \times \frac{10^6}{T^2} + 0.2365 \quad (\text{Equation 4.4})$$

where T is temperature in °K.

There is a positive offset from expected Δ_{47} values, based on our combined inorganic Δ_{47} -temperature calibration, in just over 90% of measurements of echinoid calcite. An ANCOVA test conducted indicates there is a significant difference between echinoid Δ_{47} and expected Δ_{47} based on inorganic precipitation ($F(1,108) = 15.39$, $P = 0.002$) correcting for temperature. There is however no difference in slope between the inorganic and echinoid calibrations ($F(1,108) = 0.0456$, $P = 0.8314$). We see an offset in the least squared mean value of the echinoid Δ_{47} -temperature relationship of 0.015‰ to higher Δ_{47} in comparison with our new combined inorganic calibration (Figure 4.6).

Inter-skeletal Δ_{47} values of echinoid species based range up to 0.035‰ higher than expected based on inorganic calibration (Figure 4.6). We see a much larger range in Δ_{47} observed-expected in the measured specimen of order *Camarodonta* than in specimens of other orders. In addition converse to all other echinoid inter-skeletal measurements in all groups ambulacral and inter-ambulacral calcite of the measured specimen of the order *Camarodonta* are lower Δ_{47} in comparison with expected values. We propose that this is an artifact of our method of growth temperature estimation which is based on an average of seasonal temperatures.. There is much greater seasonal SST variation in the Mediterranean Sea than at other collection localities. This may explain the greater variation in Δ_{47} of inter-skeletal calcite in the measured specimen of order *Camarodonta* assuming different inter-skeletal growth periods. Skeletal growth in warm summer periods may also explain apparent depletion Δ_{47} in comparison with expected values in the measured specimen of order *Camarodonta*.

4.2 Echinoid Δ_{47} vs. other biogenic calibration data sets

We compare our echinoid Δ_{47} dataset with biogenic datasets presented in the absolute reference frame (Figure 4.7). In order to allow direct comparison between data sets we re-process our echinoid dataset using the Santrock et al. (1985) ^{17}O correction parameters and compare with the Kelson et al. (2017) and inorganic calibration processed using the Santrock parameters. All values are corrected to acid digestion at 25°C using the AFF 0.082‰ (Defliese et al., 2015). The positive offset in Δ_{47} in echinoids is in the same direction but 0.025‰ lower than the positive offsets observed in aragonitic scleractinian corals (Kimball et al. 2014) shallow water corals (Saenger et al., 2012) and brachiopods (Came et al., 2014) at 25°C. Cephalopods (Dennis et al., 2013) are offset to lower than expected values of Δ_{47} by 0.02‰ and , an offset in the opposite direction but of similar magnitude to that observed in the echinoids (Table 5).

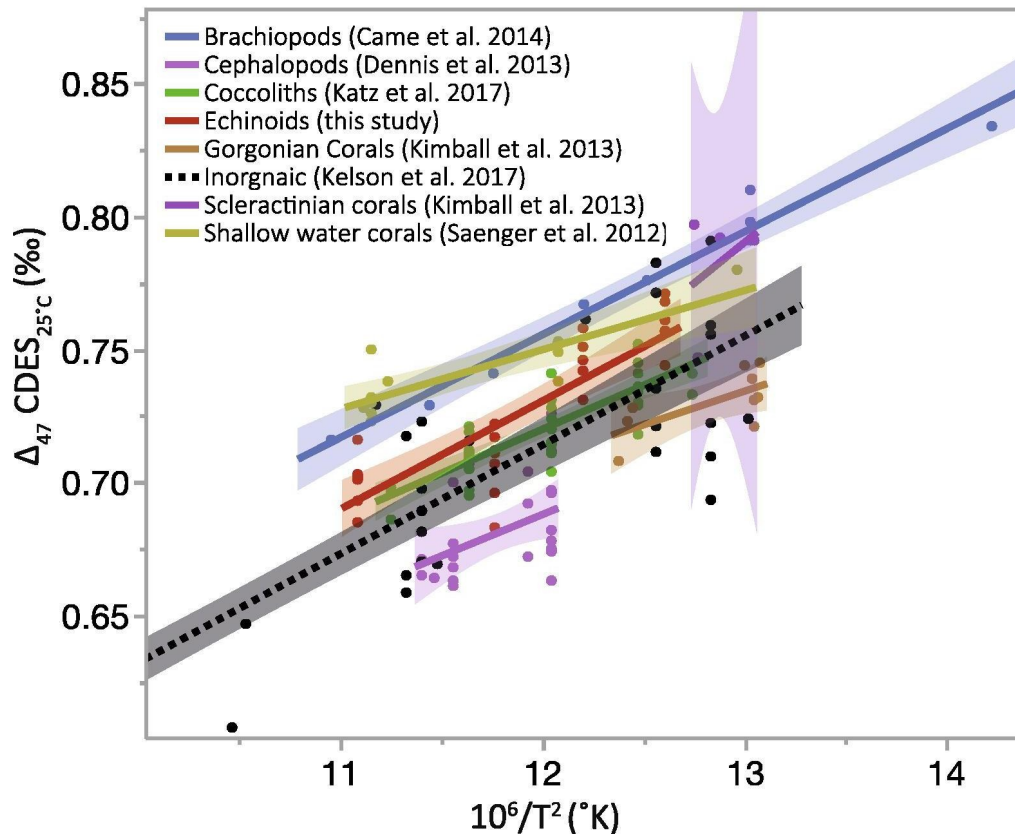


Figure 4.7 Correlation between temperature and Δ_{47} in echinoids (red line) compared to other biomineralizing groups with Δ_{47} CDES in the absolute reference scale of Dennis et al. (2011). All data are presented in the 25°C reference frame adjusting the acid fractionation factor to a common 0.082‰ (Defliese et al. 2015) using Santrock et al. 1985 ^{17}O correction parameters. Shaded errors reflect 95% confidence limits on regression.

Table 5

Overview of differences between biogenic Δ_{47} -T calibration datasets processed using the Santrock *et al.* (1985) parameters

Calibration	Equation of the Calibration Line				Higher (H) or lower (L) apparent temperature
	Gradient (a)	Intercept (b)	Δ_{47} offset (‰) ^a	Temperature offset (°C) ^b	
Brachiopods (Came et al. 2014)	0.03873	0.2908	0.041	6.73	L
Cephalopods (Dennis et al. 2013)	0.03123	0.3131	-0.027	-4.64	H
Coccoliths (Katz et al. 2017)	0.03307	0.3232	0.006	0.95	L
Echinoids (this study)	0.0407	0.2422	0.017	2.76	L
Gorgonian Corals (Kimball et al. 2013)	0.02526	0.4059	-0.006	-0.97	H
Inorgnaic (Kelson et al. 2017)	0.04097	0.2224	0.000	0.00	n/a
Scleractinian corals (Kimball et al. 2013)	0.06158	-0.0102	0.016	2.61	L
Shallow water corals (Saenger et al. 2012)	0.02226	0.4827	0.035	5.72	L

Calibration equations in the linear form $a = mx + b$ ^{a,b}offset calculated at 15°C

4.3 Sources of positive offset in Δ_{47}

4.3.1 Potential effects of sample preparation, processing and IRMS analysis

Measurement of echinoid Δ_{47} made at Imperial College following similar laboratory procedure as Kluge et al. (2015) enables us to discount difference in sample processing and IRMS analysis as a potential cause in deviation of echinoid Δ_{47} from inorganic values. Most analysis of Kluge et al. (2015) and all analysis of Kelson et al. (2017) were acidified at 90°C, the acidification temperature used in this study. This removes the complication of accurately determining the difference in acid fractionation factor between 90 and 25°C in comparing inorganic and biogenic calibrations. Our study also uses the same acid digestion technique, purification by a static PPQ trap without helium flow at -35°C, ¹⁷O correction parameters in raw data processing and carbonate standards for translation into the absolute reference frame as the inorganic calibration of Kluge et al. (2015). This enables us to make a more meaningful comparison of inorganic and biogenic datasets.

There is no systematic offset to higher Δ_{47} in ambulacral and interambulacral elements drilled with a low speed dental drill in comparison with bulk test samples powdered with a pestle and mortar (Table 2). It may therefore be concluded that sample preparation by low speed drilling does not result in resetting of calcite Δ_{47} .

4.3.2 Effect of mineralogy

Echinoderm skeletal elements are composed of Mg-enriched calcite, content ranging between 3.0 - 18.5 mol% magnesium (Clarke and Wheeler, 1917; Chave, 1954; McClintock et al., 2011) with up to 43.5 mol% “proto-dolomite” measured in axial zones of echinoid teeth (Schroeder et al., 1969).

Equilibrium constants for the equilibrium exchange reaction $M^{13}C^{16}O_3 + M^{12}C^{18}O^{16}O_2 \rightleftharpoons M^{13}C^{18}O^{16}O_2 + M^{12}C^{16}O_3$, where M is Ca or Mg in calcite, dolomite and aragonite have been calculated to be relatively constant with crystal chemistry. The equilibrium constant for the above reaction is 0.004‰ lower in dolomite than in calcite (Schauble et al., 2006). This deviation equates to differences in mineral Δ_{47} one order of magnitude smaller and in the opposite direction to observed Δ_{47} excesses in echinoid high-Mg calcite. Mineral specific differences in isotopic fraction during phosphoric digestion have also been predicted (Guo et al., 2009; Tripathi et al., 2015). At 25°C theoretically predicted $\Delta_{47\text{calcite}} - \Delta_{47\text{dolomite}} \approx 0.022\text{‰}$, a deviation of the same order of magnitude but again in the opposite direction to differences observed between echinoid Δ_{47} and inorganically precipitated calcite (Guo et al., 2009; Tripathi et al., 2015) (Figure 4.8).

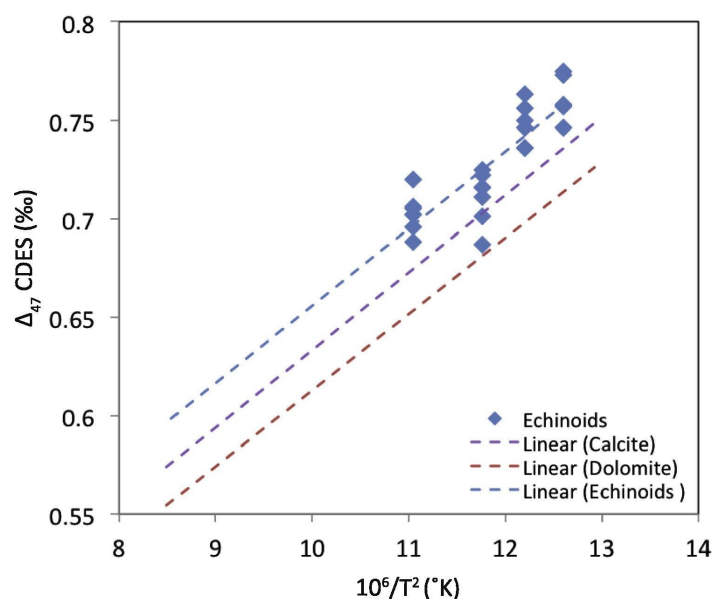


Figure 4.8 Echinoid Δ_{47} -temperature relationship in comparison with theoretical calcite and dolomite (Guo et al. 2009)

We therefore conclude that the discrepancy in the biogenic calibration of the echinoids does not relate to mineralization as high-Mg calcite. This is further supported by the lack of offset between benthic forams measured by Evans et al. (2018), also composed of high Mg-calcite,

and the combined inorganic calibration of Kluge et al. (2015) and Kelson et al. (2017) (Figure 4.6).

4.3.3 Effects of CO₂ source and diffusion into calcifying sites

Echinoid biomineralization occurs in a vacuole enclosed in a syncytial pseudopodium composed of skeletal cells (sclerocytes), a membrane envelope produced by many cells. The mineralization site is thus isolated from the environment, sclerocytes controlling of ion balance and liquid volume (Wilt, 2002; Politi et al., 2004). Skeletal tissue is separated from the external medium by an epidermis and the inner medium (body cavity) by a mesothelium.

The process of diffusion of CO₂ dissolved in seawater across the calicoblastic membrane into the calcifying fluid has been suggested as a potential mechanism causing kinetic fractionations in Δ_{47} in corals (Thiagarajan et al., 2011; Saenger et al., 2012). Thiagarajan et al. (2011) calculated resultant fractionations of liquid phase diffusion amounting to 1.6‰ in $\delta^{18}\text{O}$ and +0.036‰ for Δ_{47} (Figure 4.9). The positive offset in Δ_{47} is similar to our observations in the echinoids, however we observe a correlated enrichment in $\delta^{18}\text{O}$ in contrast to depletion (Figures 4.3, 4.9). Stump et al. (2012) demonstrate that the outer epidermis in echinoid larvae is semi-porous, allowing seawater DIC to pass directly and to flood the extracellular compartment surrounding calcifying sclerocytes. This may contribute to the lack of a diffusive signature in echinoid $\delta^{18}\text{O}$ and Δ_{47} .

Stable isotopic data from Courtney and Ries (2015) illustrates that increasing pCO₂ affects the correlation between $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ in echinoid calcite. At higher pCO₂ concentrations we observed a steeper correlation in the $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ relationship. Echinoids lack a structurally complex respiratory system, where CO₂ transport is facilitated by perivisceral coelomic fluids, the fluids composing the main body cavity of the echinoid (Weber, 1968). We propose that more positive $\delta^{13}\text{C}$ at increased pCO₂ may occur as a result of increased diffusion of environmental CO₂ into calcifying sites mixing linearly with lighter respiratory CO₂ produced at a constant rate independent of cellular pCO₂.

4.3.4 Kinetic effects during hydration/hydroxylation

Intra-specimen correlation between $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ reported here (Figure 4.2) has been previously documented. Linearly correlated variations in skeletal $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ in cidaroid

urchins are purported to relate to kinetic effects during the hydration and hydroxylation of CO_2 (McConnaughey, 1989a; McConnaughey, 1989b) calculated to be the rate-limiting step in inorganic carbon speciation (Johnson, 1982).

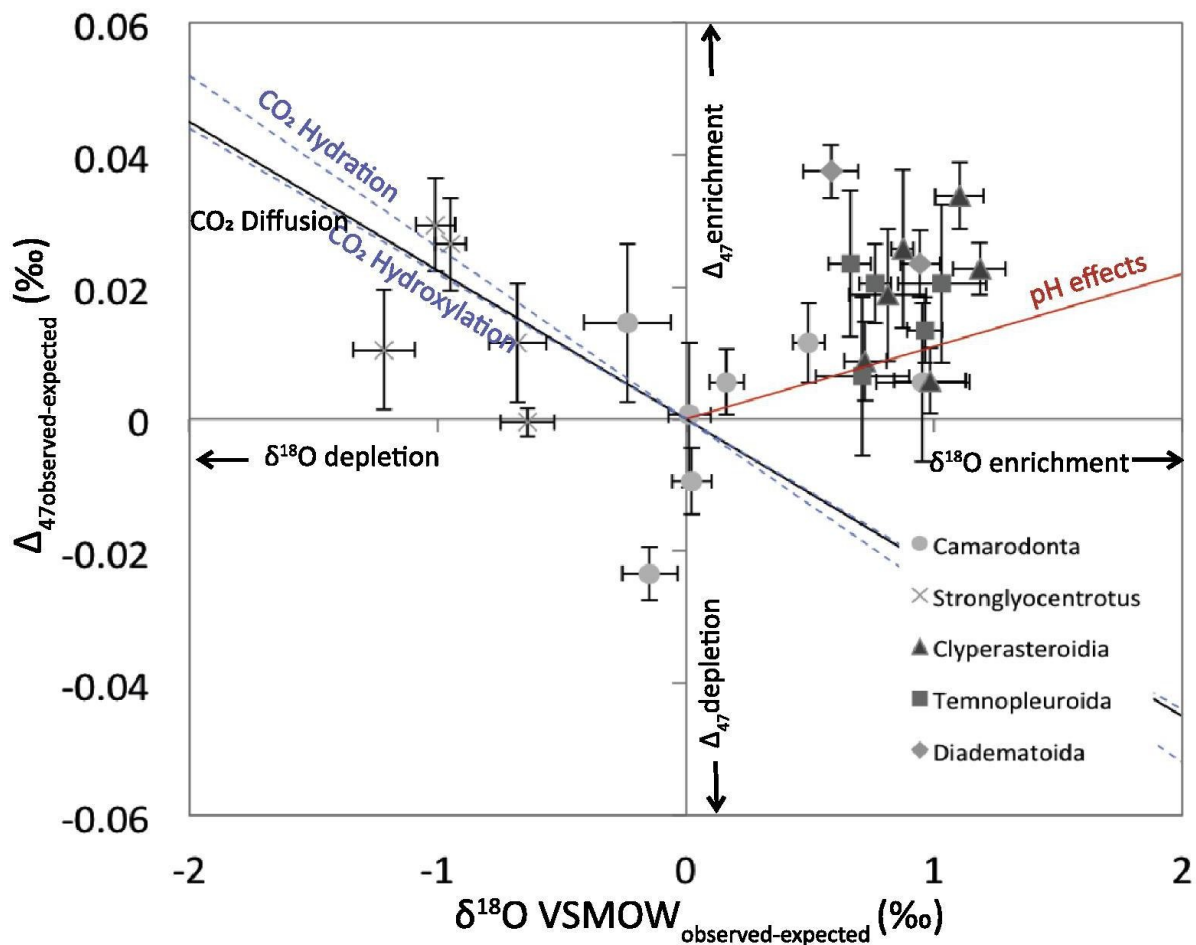


Figure 4.9 Correlation between fractionation in $\delta^{18}\text{O}$ and Δ_{47} in echinoids. Vector trajectories for effects of different processes on stable isotope signatures such as pH effects (Tripathi et al. 2015), CO_2 diffusion (Thiagarajan et al. 2011), CO_2 hydration and hydroxylation reactions (Guo, 2008). Y-error bars represent propagated error on calculated expected Δ_{47} based on calibration error, error in growth temperature and standard error on Δ_{47} observed. X-error bars represent propagated error on expected $\delta^{18}\text{O}$ based on error in $\delta^{18}\text{O}_{\text{sw}}$ at growth location, error on $\delta^{18}\text{O}$ calibration and observed $\delta^{18}\text{O}$ measured at Imperial College London.

If kinetic effects during hydroxylation and hydration of CO_2 purported to offset biogenic calcite $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ from equilibrium precipitation have a measurable effect on echinoid Δ_{47} , we might expect to see correlated relationships between these variables and echinoid Δ_{47} both inter-skeletally and between species. Hydration affects the isotopic composition of CO_2 diffusing into the calcifying site, thus the overall composition of the DIC. Likewise dehydration followed by degassing also effects the overall composition of the DIC. The hydration/dehydration of CO_2 has been demonstrated to occur at the same rate for Δ_{47} as $\delta^{18}\text{O}$ suggesting disequilibrium in one system will also manifest in the other (Affek, 2013) and is

postulated to be a contributory factor in measured positive Δ_{47} offsets in surface corals (Saenger et al., 2012). Kinetic effects during hydration/dehydration result in enrichment in Δ_{47} and associated depletion $\delta^{18}\text{O}$. This is converse to the enrichment in both $\delta^{18}\text{O}$ and Δ_{47} observed in most echinoid data (Figure 4.9). Inter-skeletal calcite from the cold-water echinoid *Stronglyocentrotus* grown at 8.5°C does however show more depleted $\delta^{18}\text{O}$ and enriched Δ_{47} compared to inorganic calcite equivalent, implying the potential action of kinetic effects during precipitation particularly in $\delta^{18}\text{O}$. The manifestation of kinetic effects is dependent on the ratio of the rate of precipitation to the rate of isotopic equilibration of the DIC pool. The timescale of equilibration of DIC in solution is longer at lower temperature (Zeebe and Wolf-Gladrow, 2001) thus it is a reasonable assumption that kinetic effects may play a role in observed “vital effects” in echinoids growing fast at lower water temperatures.

4.3.5 pH effects

The fluid composition of isolated environments such as calcification sites in echinoids has the potential to yield significant deviation in fluid composition from seawater values, including variable pH and ion concentration. This allows for significant variation in the DIC pool from with the mineral precipitates compared to external seawater.

The calcifying fluid DIC solution consists of carbonic acid (H_2CO_3) dissolved carbon dioxide ($\text{CO}_{2(\text{aq})}$), bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) ions. Theoretical modelling by Hill et al. (2014) indicates each DIC species has a distinct equilibrium clumped isotope signature, $\Delta_{47}(\text{H}_2\text{CO}_3) > \Delta_{47}(\text{HCO}_3^-) > \Delta_{47}(\text{equilibrium calcite}) > \Delta_{47}(\text{CO}_3^{2-})$, thus speciation within the parent DIC pool has the potential to affect clumped isotope composition of precipitating carbonate minerals (Hill et al., 2014). The difference between $\Delta_{47}(\text{HCO}_3^-)$ and $\Delta_{47}(\text{CO}_3^{2-})$ at 25°C is predicted to be 0.018‰ (Guo et al., 2008) to >0.033‰ (Hill et al., 2014). The effect of varying pH alters the partitioning of DIC species according to equilibrium constants pK_1 and pK_2 . A decrease in pH would shift equilibrium to the left favouring the formation of H_2CO_3 over HCO_3^- (Eqn. 5) and HCO_3^- over CO_3^{2-} (Eqn. 6) (Millero et al., 2006; Hill et al., 2014). Calculations of the Δ_{63} , defined as the per mil deviation in the abundance of all CO_3^{2-} isotopologues from the abundance predicted by random mixing, for the composite DIC pool, indicates increased Δ_{63} with decreases in pH. This effect is amplified at increased salinities ($S = 35,50$) (Hill et al., 2014).



It has been hypothesized that pH effects could explain observed variations in Δ_{47} and $\delta^{18}\text{O}$ in deep-sea corals (Thiagarajan et al., 2011). Coral calcification is associated with an increase of the pH of the calcifying fluid (pH_{cf}) from seawater pH ($\text{pH}_{\text{sw}} \sim 8.1$) (Marion et al., 2011). The pH_{cf} in corals has been investigated using the boron isotopic pH proxy and has been found to range between 8.3 and 8.5 (McCulloch et al., 2012; Holcomb et al., 2014; Sutton et al., 2017) while microelectrode measurements of scleractinian coral species indicate they elevate pH_{cf} by 1-2 units relative to pH_{sw} (Al-Horani et al., 2003; Ries, 2011). The effects of pH_{cf} in corals thus tend to a general decreases in Δ_{63} (Fig. 4.10) (Hill et al. 2014). Using the boron isotope proxy, tropical

echinoids pH_{cf} has been shown to be only 0.1 units greater than pH_{sw} , whereas temperate echinoids pH_{cf} tends to be 8.0, lower than pH_{sw} (Sutton et al., 2017). Assuming the Δ_{63} signature of the DIC is directly inherited during mineral precipitation i.e. precipitation occurs fast enough that calcite crystals do have time to come to internal bulk equilibrium, a decrease in pH_{cf} in echinoids is in agreement with higher than expected Δ_{47} of echinoid calcite (Hill et al., 2014) (Figure 4.11). It must be noted however that based on the model of Hill et al. (2014) the offset in Δ_{47} from equilibrium at pH values ~ 8.6 is at its maximum 0.009‰, almost 3 times lower than Δ_{47} enrichment observed in the echinoids.

Experimental results however indicate a difference in Δ_{47} (thus Δ_{63}) between CO_3^{2-} and HCO_3^- of $0.063 \pm 0.006\text{‰}$ (Tripathi et al., 2015), twice that of theoretical predictions, suggesting greater magnitude effects of variations in pH_{cf} on Δ_{47} . The observed slope of Δ_{47} observed-expected against $\delta^{18}\text{O}$ observed-expected, excluding anomalous *Stronglyocentrotus* samples, is remarkably similar to the observed slope of regression (0.011 ± 0.001) of synthetic witherite precipitation at variable pH (Figure 4.10) (Tripathi et al. 2015).

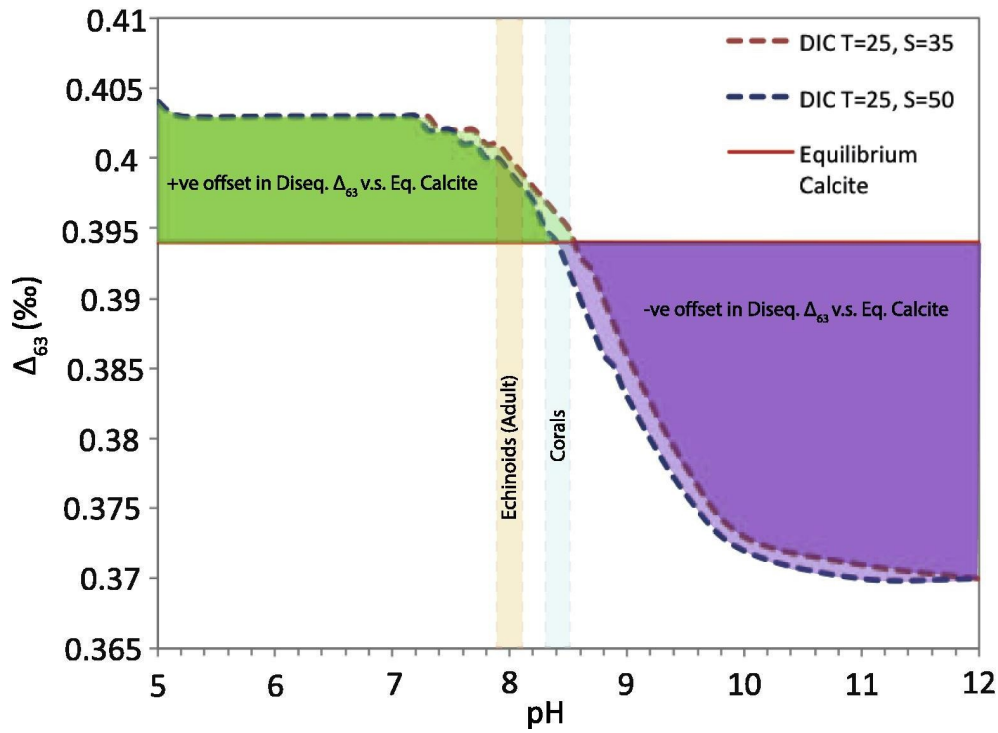


Figure 4.10 pH dependence of Δ_{63} of DIC (Hill et al. 2014). Shaded boxes show pH_{cf} echinoids (Sutton et al. 2017) and corals (Al-Horani et al. 2003, Ries 2011).

It may therefore be concluded that pH effects relating to direct inheritance of the Δ_{63} signature from the DIC may contribute to but may not be the sole cause of enrichments in echinoid Δ_{47} from expected values. The potential for observable pH effects is greater at increased salinities (Tripathi et al. 2015, Hill et al. 2014).

4.3.6 Precipitation/calcification rate

Echinoid $\Delta^{18}\text{O}$ ($\Delta^{18}\text{O} = \delta^{18}\text{O}_{\text{e}} - \delta^{18}\text{O}_{\text{DIC}}$) is weakly negatively correlated with calcification rate (Courtney and Ries, 2015). Likewise in inorganic precipitation experiments, oxygen isotope fractionation has been demonstrated to depend on precipitation rate (R) ($1000 \ln \alpha_{\text{calcite-water}} = 1.094 \log R + 30.87$ (Dietzel et al. 2009)). As echinoids measured as part of this study were mostly collected live from the benthos, measurement of precipitation rate and comparison with Δ_{47} offset has not been possible. Previous studies however indicate that echinoids are capable of rapid calcite precipitation, the inner stereom of snapped spines calcifying at a longitudinal rate of 125 $\mu\text{m/day}$ (Gorzela 2011). Courtney and Ries (2015) give an approximation of growth rate of the bulk shell reporting up to a 0.504% d^{-1} change in buoyant weight over a 60 day period in adult *Echinometra viridis* while juvenile *Tripeustes gratilla* calcify at up to 2.75% d^{-1} . If calcite crystals precipitate slowly enough to reach internal bulk equilibrium, their Δ_{47} will remain independent of the Δ_{63} of the DIC and by extension of the pH of the parent solution (Tripathi et al. 2015). This may explain why correlated $\delta^{18}\text{O}$ - Δ_{47} effects

relating to pH of the calcifying fluid appear to manifest in rapidly calcifying echinoids but not in other groups.

Crystal growth models such as the growth entrapment model (Watson and Liang, 1995; Watson, 2004; Gabitov, 2013) and surface kinetic models (DePaolo, 2011) link net precipitation rate to potential disequilibrium in isotopic signatures when growth rates are high. Ion-by-ion growth models (Watkins and Hunt, 2015), assuming attachment and detachment rate coefficients for HCO_3^- and CO_3^{2-} isotopologues, are useful in explaining disequilibrium signatures in biological carbonates considering predicted differences in bond ordering between the two ions. Experimental results based on inorganic precipitation under varying conditions however do not capture differences in Δ_{47} as a result of growth effects eg precipitation rate (Tang et al., 2014) whilst others indicate temperature dependent Δ_{47} - $\delta^{18}\text{O}$ co-variance indicating additional fractionation in fast growing minerals that effects in $\delta^{18}\text{O}$ but not Δ_{47} (Affek and Zaarur, 2014).

4.3.7 Other potential effects

A notable feature of echinoid biomineralization is crystal formation via an initial amorphous calcium carbonate (ACC) phase on an organic matrix. ACC is formed as a transient precursor phase both in echinoderms (Beniash et al., 1997; Politi et al., 2004) and some bivalve molluscs (Weiss et al., 2002). The appearance of this biomineralization mechanism on two different branches of the phylogenetic tree indicates that, although difficult to diagnose, it may be widespread amongst invertebrates (Raz et al., 2003). The formation of ACC and hindrance of conversion to more stable crystalline phases requires unique conditions such as the creation of a microenvironment disfavoring organization of nuclei (Addadi et al., 2003) or the introduction of additives for example ions or organic macromolecules add that interfere with crystal growth (Raz et al., 2003). Despite our lack of understanding of exactly how ACC forms (Addadi et al., 2003) it is conceivable that either of these mechanisms may act to promote a unique Δ_{47} signature independent of environmental conditions.

The mechanism by which ACC is converted into calcite remains uncertain (Addadi et al., 2003). Further knowledge of how this occurs is important as it may determine whether the potentially unique Δ_{47} composition in ACC is inherited. If ACC is converted to calcite by dissolution and re-precipitation in interaction with water in an open system then Δ_{47} composition of ACC will be lost. If however this conversion occurs under closed conditions, i.e.

within a closed cellular compartment, or without breaking of C-O bonds, preservation of a unique non-environmentally representative Δ_{47} signal may still occur. In urchin larval spicules the growing spicule is initially composed of ACC prior to the propagation of a single crystal through the ACC phase without evidence of an identifiable crystallisation front (Beniash et al., 1997). This occurs within the isolated environment of the confining vesicle so the ACC is not in contact with the surrounding aqueous solution. Likewise CaCO_3 is delivered to the spicule forming compartment as pre-formed ACC, also confined to vesicles thus preventing interaction with the surrounding environment (Beniash et al., 1997). Stabilisation to calcite is thought to occur by breakdown, conversion or rearrangement of macromolecules (Raz et al., 2003). The occurrence of this process in isolation favours preservation of the initial Δ_{47} signal of ACC.

Matrix macromolecules in the presence of magnesium ions have been demonstrated to be responsible for the formation and stabilization of ACC in sea urchin larval spicules (Raz et al., 2003). An experimental study by Wang et al. (2009) indicates that Mg content in biogenic ACC is regulated by macromolecules including carboxylated (acidic) proteins. Mg/Ca ratios appear to be controlled by a predictable dependence on binding properties of organic molecules related to electrostatic topology. It is reasonable therefore to assume that electrostatic topology of the organic mineralization matrix in groups such as echinoids could have an influence in the adsorption of CO_3^{2-} and/or HCO_3^- isotopologues into the calcite surface thus impacting Δ_{47} . We however do not observe any relationship between offsets in Δ_{47} and Mg/Ca ratio in echinoid inter-skeletal elements as reported by Webber (1969). Kimball et al. (2015) measured the clumped isotope composition of Gorgonian corals that, like the echinoids, form high-Mg calcite on an organic matrix. The gorgonians display an offset in Δ_{47} opposite in polarity to that observed in the echinoids (Kimball et al., 2015) thus it is difficult to reconcile the two with the same mechanism. It is however notable that both display discernable offsets from the inorganic Δ_{47} calibration in comparison with equilibrium precipitators such as coccoliths (Katz et al., 2017) and benthic forams (Evans et al., 2018).

4.4 Perspective for the use of echinoderm Δ_{47} in paleoclimate reconstruction

Echinoderms offer good targets for paleoclimate reconstruction as a result of their relatively high abundance and ubiquitous distribution in most marine facies (Lebrato et al., 2010) and long fossil record, dating back to the Early Cambrian (Bottjer et al., 2006). The echinoderm origin of sediment particles can be recognised by their characteristic microstructure. They are

an important component of Cenozoic carbonate systems and can compose between 5-30% of sediment particles (Kroh and Nebelsick, 2009). Echinoids are particularly common in Oligo-Miocene carbonates, clypeasteroids forming mass occurrences in shore face sequences, which can be up to several metres in thickness (Nebelsick and Kroh, 2002).

The high-Mg calcite composition of echinoderms is thermodynamically metastable under environmental conditions, however recrystallization during early diagenesis is often inhibited by organic and inorganic linings (Berner, 1966; Weber, 1969). Cement coating plays an important role in preservation, forming so called “crystal caskets” that prevent ion exchange with echinoderm calcite and surrounding pore water (Dickson, 2001). Dickson et al. (1995, 2004) report examples dating back to the Silurian where echinoderm high Mg-calcite has been preserved enabling the application of stable isotope techniques. Furthermore diagenetic recrystallization is easier to detect in organisms composed of high-Mg calcite as this is rapidly converted to low-Mg, low-Sr calcite with no continuum in Mg/Ca-Sr/Ca space between well-preserved and poorly preserved samples. Mg/Ca and Sr/Ca ratios can thus been used to identify recrystallized samples (Evans et al., 2018).

Variability in fractionation of $\delta^{18}\text{O}$ both inter-skeletally and between individuals combined with uncertainties in the $\delta^{18}\text{O}_{\text{sw}}$ of ancient oceans however serves to complicate paleotemperature reconstruction based solely on stable oxygen isotopic composition of skeletal calcite. Previous studies indicate strong vital effects in $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ with high inter-specific, inter-specimen and inter-skeletal variation. This variation is controlled by a variety of factors, largely genetic but also including temperature, depth of growth, food supply and ambient $\delta^{13}\text{C}$ - level and is thus non-predictable (Weber and Raup, 1966a; Weber and Raup, 1966b; Weber, 1968). The most significant correlation of $\delta^{18}\text{O}$ with temperature is observed in test calcite ($r^2 = -0.34$, $N = 153$) (Weber and Raup, 1966b).

The clumped isotope composition of echinoid calcite is shown to be homogenous inter-skeletally (Figure 4.4). Additionally, although the clumped isotopic composition of echinoid calcite shows a positive small offset from expected values based on inorganic precipitation experiments, the positive Δ_{47} offset is consistent among echinoid groups. The offset in echinoid Δ_{47} may thus be corrected for either by deducting 0.015‰ from echinoid Δ_{47} values prior to applying our inorganic Δ_{47} -T calibration. This offset, although small, is statistically robust at 0.0146 ± 0.0042 ‰. The error calculated on this offset using a least squared means student t-test adds only a $\pm 0.31^\circ\text{C}$ uncertainty on the total propagated temperature error of

our inorganic calibrations (Kluge et al., 2015; Kelson et al., 2017). Moreover in contrast to echinoid $\delta^{18}\text{O}$ calibration, the offset in echinoid Δ_{47} from the inorganic calibration is constant across the range of measured temperatures (i.e. both the echinoid and inorganic calibrations have indistinguishable slopes). This predictable offset makes echinoid Δ_{47} a more reliable temperature proxy than echinoid $\delta^{18}\text{O}_{\text{VPDB}}$. Vital effects in echinoid $\delta^{18}\text{O}$ however still remain a barrier to the use of combined Δ_{47} and $\delta^{18}\text{O}_{\text{VPDB}}$ to back calculate $\delta^{18}\text{O}_{\text{sw}}$. The positive 0.015‰ offset of echinoid calcite from the organic calibration is only demonstrated here within a 8.5°C to 28°C range of growth temperature, and thus applications beyond this range requires extrapolation of the data or further study to validate the application of the clumped isotope proxy to echinoids growing at very low sea temperatures (1-2°C).

5) CONCLUSIONS

This study is the first to systematically measure echinoid Δ_{47} . The inter-skeletal variation in Δ_{47} is insignificant. We find however a positive offset of 0.014‰ in Δ_{47} relative to the inorganic calibration of the clumped isotope thermometer, corresponding to approximately a 4.5°C underestimation of precipitation temperature at 15°C. Although small the 4.5°C underestimate in growth temperature observed based on clumped isotopic composition is consistent across echinoid orders, both regular and irregular. The magnitude of positive Δ_{47} offset in the echinoids is approximately half that of the positive Δ_{47} offsets reported in brachiopods and shallow water corals (Saenger et al., 2012; Came et al., 2014). This offset is however statistically significant and cannot be explained by inter-laboratory differences in calibration or mineralogical differences. Four of five echinoid species measured in this study show enrichment in Δ_{47} of echinoid calcite associated with corresponding enrichment in $\delta^{18}\text{O}$. Neither kinetic effects during hydroxylation of CO_2 nor isotopic fractionation during CO_2 diffusion into calcifying sites can explain this pattern. Enrichments in Δ_{47} and $\delta^{18}\text{O}$ of the magnitude observed may however correspond to pH effects on DIC speciation in the calcifying fluid in association with rapid precipitation rate and potential effects relating to ACC precipitation on an organic matrix. Depletion in $\delta^{18}\text{O}$ and enrichment in Δ_{47} of skeletal elements of echinoid *Stronglyocentrotus* may also imply potential kinetic effects during rapid precipitation at very low growth temperatures.

Based on our results, we suggest that the echinoids may be added to a growing list of calcifying groups that show evidence of an offset in clumped isotope composition in

comparison with inorganic precipitation experiments. This offset is however constant across growth temperature range of species measured and can thus easily be corrected. This suggests that Δ_{47} in echinoids may be a more reliable temperature proxy than $\delta^{18}\text{O}_{\text{VPDB}}$. The addition of a further group to the growing list of organisms showing statistically distinguishable “vital effects” in Δ_{47} is proof that these effects are more widely spread than at first conceived.

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Chapter 5: The clumped ^{13}C - ^{18}O isotopic composition of modern cephalopod carbonate: a further example of taxonomic non-equilibrium effects in the clumped isotope proxy

ABSTRACT

Ammonites were abundant in Mesozoic and are important zone fossils. The ^{13}C - ^{18}O clumped isotope compositions of cephalopods, such as ammonites and belemnites, may thus be valuable in reconstructing Mesozoic sea temperatures and $\delta^{18}\text{O}_{\text{sw}}$. Our measurements of the clumped isotopic composition of modern cephalopods (*Nautilus Pompilius*, *Nautilus Macromphalus* and *Nautilus Belauensis*) however indicate a significant “vital effects” in clumped isotopic composition corresponding to on average a 10°C overestimate in shell formation temperature.

Intra-shell clumped isotopic composition (Δ_{47}) is correlated with $\delta^{18}\text{O}$ shell carbonate. We further show a decrease in Δ_{47} from juvenile to adult septa. This trend does not correspond with changes in surface seawater temperature or temperature variation relating vertical migration within the water column. The clumped isotope composition of most recently formed septa yield growth temperatures $3\text{--}8^\circ\text{C}$ above the upper temperature limit of survival of the organism (27°C). We posit non-equilibrium clumped isotope signatures most likely occur as a result of variations in salinity in cameral fluid within nautili chambers however may relate to dehydration of HCO_3^- and/or isotopic effects relating to CO_2 diffusion out of the extrapallial fluid (EPF).

Significant offsets in Δ_{47} of nautili from expected values and associated subtle non-equilibrium $\delta^{18}\text{O}$ signatures have important implications for paleoclimate reconstruction using the stable isotopic composition of cephalopod aragonite. Non-realistic clumped isotope calculated temperatures of fossil specimens such as ammonites, with minimal evidence of diagenetic alteration, might in part be due to previously un-diagnosed “vital effects”.

1) INTRODUCTION

Cephalopoda is an ancient, highly specialized class of Molluscs. They are thought to have evolved during the Cambrian at approximately 530 Ma (Kröger et al., 2011). The long fossil record, wide distribution and high abundance with preservation of durable skeletal material,

primarily calcium carbonate, make cephalopods an excellent target for stable isotope geochemistry.

Cephalopods, specifically ammonites (Stahl and Jordan, 1969; Dennis et al., 2013; Stevens et al., 2015) and belemnites (Bowen, 1966; Stevens and Clayton, 1971; Podlaha et al., 1998; Korte et al., 2009; Price and Passey, 2013), have been extensively used in paleotemperature reconstruction using stable oxygen isotopes and more recently clumped isotopes (Dennis et al., 2013). These taxa became extinct around 65 Ma ago (Keller, 2000) thus isotopic temperature calibration has been carried out using modern genera such as Nautili and Sepia.

For this approach to be valid (1) there must be no shell diagenesis; (2) there should be no biogenic fractionation known as “vital effects”; (3) ecological factors and ontogenetic changes that affect the lifestyle of the animal and thus isotopic composition of the shell must be accounted for (Rexfort and Mutterlose, 2006) and (4) an *a priori* assumption of $\delta^{18}\text{O}_{\text{sw}}$ must be made. Prior assumption of $\delta^{18}\text{O}_{\text{sw}}$ is not always possible as it varies both geographically and through time, and is particularly affected in semi-isolated basins and when sea level changes are forced by changes in global ice volume.

Nautilus and Sepia shells were widely believed to precipitate in oxygen isotope equilibrium with seawater. Recent studies however indicate vital effects in the oxygen isotopic composition of Nautilus shells with inconsistent $\delta^{18}\text{O}$ values between outer shell walls and contemporaneously secreted septa (Ohno et al., 2014). Variations in $\delta^{18}\text{O}$ between embryonic and early septa have been purported to reflect changes in temperature on vertical migrations during the lifespan of the organism, however, $\delta^{18}\text{O}$ of septa also co-varies with cameral liquid $\delta^{18}\text{O}$ in living Nautilus (Oba et al., 1992). As shell $\delta^{18}\text{O}$ carbonate is dependent on temperature and $\delta^{18}\text{O}$ fluid composition, the stable oxygen isotope proxy may be considered insufficient to estimate habitat conditions and thus lifestyle of modern and ancient nautili (Oba et al., 1992).

Carbonate clumped isotope paleothermometry is a temperature proxy based on the thermodynamically dependent relative abundance of $^{13}\text{C}\text{-}^{18}\text{O}$ bonds within the carbonate crystal lattice, quantified by the metric Δ_{47} (Eiler and Schauble, 2004; Ghosh et al., 2006; Eiler, 2007). This is based on the self-reaction isotope exchange equilibrium $\text{M}^{13}\text{C}^{16}\text{O}_3 + \text{M}^{12}\text{C}^{18}\text{O}^{16}\text{O}_2 \rightleftharpoons \text{M}^{13}\text{C}^{18}\text{O}^{16}\text{O}_2 + \text{M}^{12}\text{C}^{16}\text{O}_3$ (where M is a metal such as Ca) (Schauble et al., 2006). The $\delta^{18}\text{O}$ carbonate therefore is independent of the oxygen isotope composition of the parent

fluid. The clumped isotope thermometer is also considered to be relatively free of “vital effects” that plague traditional oxygen isotope paleothermometry. Groups with significant non-equilibrium fractionation of $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ appear free of vital effects in their clumped isotopic composition (Tripathi et al., 2010; Thiagarajan et al., 2011; Grauel et al., 2013; Katz et al., 2017) empirical calibrations being in good accordance with theoretical calibration on varying temperature (Schauble et al., 2006; Guo et al., 2009).

Dennis et al. (2013) however report a significant offset in clumped isotopic composition (Δ_{47}) of modern nautilus and sepia from the inorganic calibration of Ghosh et al. (2006). This is to date the only example of disequilibrium clumped isotope compositions in the presence of equilibrium oxygen isotope compositions. However it is unclear whether the discrepancy reported in the study by Dennis et al (2013) resulted from inadequate knowledge of growth temperatures, concerns relating to inorganic calibration of the clumped isotope thermometer or problems with methodological artefacts and/or inter-laboratory calibration. Improvements in sample measurement, data processing (Daëron et al., 2016; Schauer et al., 2016) and inorganic calibration (Bonifacie et al., 2016; Kelson et al., 2017) of the clumped isotope thermometer now make it easier to assess non-equilibrium fraction in the clumped isotope composition of biogenic carbonate.

We therefore present an assessment of the clumped isotopic composition (Δ_{47}) of modern cephalopod aragonite both addressing variations in Δ_{47} between and within individuals. We use observations of non-equilibrium precipitation in the clumped isotopic composition of cephalopods to infer how variations in biomineralization strategy may result in non-equilibrium isotopic effects during shell growth. Furthermore the findings in this study enable us to assess the utility of the isotopic composition of cephalopod carbonate as archive of past climate change.

2. METHODOLOGY

2.1 Origin of Sample used in this study and assessment of their growth environment

Samples *Nautilus Pompilius* (NLI), *Nautilus Macromphalus* (NNCS and NNCL) and *Sepia Officianalis* (SO and SO2) were provided by the Natural History Museum, London. The American Museum of Natural History provided samples of *Nautilus Macromphalus* (ANNC loan number ANMH 105621) and *Nautilus Belauensis* (ANPU loan number ANMH 105622 and

ANWA loan number ANMH 102555). Samples ANMH 105621 and 102555 have been previously analysed for oxygen isotope variability in growth bands (Linzmeier et al., 2016). *Nautilus Belauensis* (NPU), provided by Waikiki Aquarium, was captured in waters surrounding Pualu prior to transferral to an aquarium tank maintained at 22°C. Finally *Sepia Omani* (SO3) was collected on the beach at Bimma, North East Oman. Location and further details of sample collection are given in Table 1.

Growth temperatures (Table 1) are estimated from the World Ocean Database (V.2) (Locarnini et al., 2013). Nautili are known to migrate vertically within the water column inhabiting depths between 130 and 700 m, dictated by an upper temperature tolerance limits of around 25°C and implosion depths of 800 m (Dunstan et al., 2011a; Dunstan et al., 2011b). The large variation in depth habitat makes it more difficult to estimate growth temperature, however studies indicate most time spent at 175-225 m (Dunstan et al., 2011a; Dunstan et al., 2011b). Growth temperature, shown in Table 1, is therefore average seawater of temperature over the measured depth range of tagged nautili weighted towards temperatures at depths at which nautili spend most time (Dunstan et al., 2011b). We also use temperature-depth profiles from the World Ocean Database (V.2) in our analysis of the data (Locarnini et al., 2013). These are shown in supplementary data (S1).

The $\delta^{18}\text{O}$ values of seawater are estimated from the global gridded dataset of LeGrande and Schmidt (2006). As with growth temperature, growth $\delta^{18}\text{O}$ shown in Table 1 is given as a average of seawater $\delta^{18}\text{O}$ over the measured depth range of tagged nautili weighted towards temperatures at depths at which nautili spend most time (Dunstan et al., 2011b). Calculations pertaining to determining depth weighted average growth temperature are shown in supplementary data (A1). Individuals ANWA and ANPU spent either part or all of their lifespans in aquaria under well-controlled temperature conditions ($22 \pm 1^\circ\text{C}$).

Table 1. Specimen sampling details and collection location. Growth water temperature and stable oxygen isotopic composition of analysed samples

Sample information						Seawater Properties	
Individual	Genus	Species	Samples	Collection Location	Collection Details	Growth Temp (°C) ^a	Growth $\delta^{18}\text{O}_{\text{sw}}$ VSMOW (‰) ^b
NLI	Nautilus	Pompilius	NNLI	Lindeman Island, Queensland, AU	Beach Collection	19.4	0.49
NFI	Nautilus	Scrobiculatus	NNFI	Florida Islands, Solomon Islands	Beach Collection	18.39	0.44
NTB	Nautilus	Pompilius	NNTB	Taema Bank, American Samoa	Live collected at 208m depth	20.63	0.58
NNCS	Nautilus	Macromphalus	NNCS	Noumea, New Caledonia	Beach Collection, Bae Des Citrons	19.02	0.51
NNCL	Nautilus	Macromphalus	NNCL	Noumea, New Caledonia	Beach Collection, Bae Des Citrons	19.02	0.51
NSO1	Sepia	Officinalis	NSO1	Littlehampton, Sussex, UK	Beach Collection	12.04	0.28
NSO2	Sepia	Officinalis	NSO2	Littlehampton, Sussex, UK	Beach Collection	12.04	0.28
NSO3	Sepia	Officinalis	NSO3	Qalhat, Oman	Beach Collection	20.41	0.72
ANWA	Nautilus	Belauensis	ANWA	Waikiki Aquarium, Hawaii	Aquarium raised	22	-0.2
ANNC	Nautilus	Macromphalus	ANNC; ANNC1-17	Noumea, New Caledonia	Live captured July 2002	19.02	0.506
ANPU	Nautilus	Belauensis	ANPU; ANPUS1-15	Republic of Palau	Live Captured	11.93	0.618
WNPU	Nautilus	Belauensis	WNPU01-5	Republic of Palau	Live captured in Palau, transferred to Waikiki Aquarium	11.93 to 22°C for septa 1	0.618

^a Ocean temperature estimated from the NOAA World Ocean Atlas 2013 (v.2) (Locarnini *et al.* 2013) ^b Water $\delta^{18}\text{O}$ values estimated from the global gridded data set of oxygen isotope composition in water (LeGrande & Schmidt 2006) excluding ANWA and WNPU01 where water $\delta^{18}\text{O}$ was directly measured from an aliquot taken from the aquaculture tank. Error on T°C and Water $\delta^{18}\text{O}$ are given in Appendix 1 (Tables 1A and 2A)

2.2 Sampling for isotopic analysis

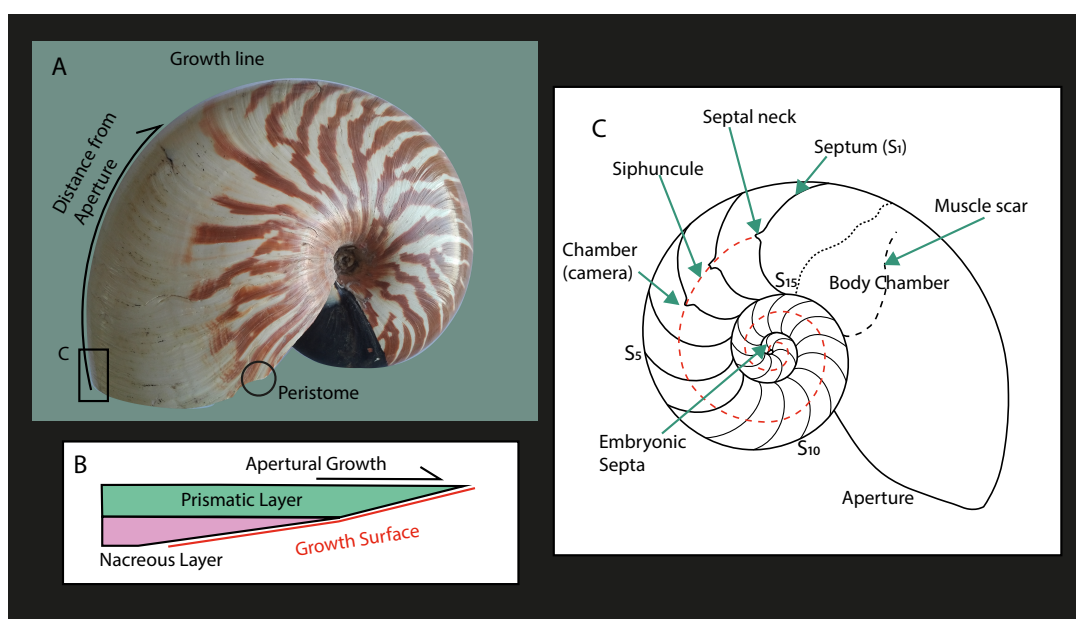


Figure 5.1 Shell morphology and sampling of Nautilus shells a) outer shell measurements b) cross section across the peristome away from direction of apertural growth c) medial cross section showing septa sampled, sphuncule, body chamber and aperture.

Nautili shells consist of an outer prismatic layer and an inner nacreous layer. In samples NLI, NFI, NTB NNCS and NNCL we sampled the outer prismatic layer from the peristome (Figure 5.1a,b) using a dental drill at low speed. The outer prismatic layer of sample NPU was also sampled at 5 locations around the outer rim temporal series of the specimen (Figure 5.1). Samples were taken parallel to growth lines. Bulk-carbonate measurements of selected septa, numbered from most recent (1) to oldest, as shown in Figure 5.1c were also performed. In contrast sample ANWA, only a fragment of shell due to the small nature of the individual, was crushed gently using a pestle and mortar. Sepia shells were sampled in the dorsal shield region also using the dental drill on low speed.

2.3 Stable isotope measurements

Isotopic analyses were carried out in the Qatar Stable Isotope Laboratory at Imperial College London. Typical sample sizes were in the range of 5-6 mg per replicate. Measurement of ^{13}C - ^{18}O ordering in sample carbonate is achieved by measurement of the relative abundance of the $^{13}\text{C}^{18}\text{O}^{16}\text{O}$ isotopologue in acid evolved CO_2 .

Analyte CO_2 was measured on two dual inlet Thermo MAT 253 mass spectrometers (Santa Maria and Pinta) as described in **chapter 2**. The reference gas, in both machines is the same high purity CO_2 , $-36.97 \delta^{13}\text{C}_{\text{VPDB}}$, $8.6 \delta^{18}\text{O}_{\text{VSMOW}}$. Measurements comprise 8 acquisitions each

with 7 cycles with 26 s integration time. A typical acquisition time is 20 minutes corresponding to a total analysis time of 2.5-3 h.

This study uses two different CO₂ purification methods, both yielding comparable Δ_{47} after data correction. Approximately 50% of sample replicates were acidified individually in 105% orthophosphoric acid at 90°C for 10 minutes on a manual vacuum line following (McCrea, 1950) and (Swart et al., 1991). Analyte CO₂ was then purified by using manual cryogenic purification through a series of water traps and a static Porapak Q packed U-trap, without the use of helium as a carrier gas (Dennis and Schrag, 2010; Davies and John, 2017; **chapter 2**). These samples were then measured on mass spectrometer Santa Maria. The other 50% of samples were measured on our new automated clumped isotope measurement system (the IBEX: Imperial Batch Extraction system) as described in **chapter 2**. The IBEX was developed at Imperial College London and is manufactured and distributed by Protium^{ms}.

2.4 Data analysis and reduction

All calculations and corrections are carried out in the freely available stable isotope management software Easotope (John and D. Bowen, 2016) (www.easotope.org). The mass 47 beam of CO₂ is composed primarily of ¹⁸O¹³C¹⁶O and thus we can use the intensity of signal on mass 47 to determine ¹³C-¹⁸O bond abundance in CO₂. The abundance on masses 47, 46 and 45 are normalized by the abundance on mass 44 ($R^i = m_i/m_{44}$). From this Δ_{47} can be calculated as:

$$\Delta_{47} = \left(\frac{R^{47}}{R^{47*}} - 1 \right) - \left(\frac{R^{46}}{R^{46*}} - 1 \right) \left(\frac{R^{45}}{R^{45*}} - 1 \right) \times 1000$$

(Equation 5.1)

where R^* gives the abundance of each isotopologue of a gas with the same bulk isotopic composition as R but with a stochastic distribution amongst isotopologues (Affek and Eiler, 2006).

Raw Δ_{47} is corrected in three steps. In order to correct for non-linearity of the mass spectrometer we carry out a pressure baseline correction. External “peak shape” scans are run daily, at partial pressures equating to mass intensities between 12 kv and 17 kv on the mass 44 beam. The acceleration range of the background “pick” selected is then used for correction following (Bernasconi et al., 2013) as described in John and Bowen (2016). Further

details of this correction are given in **chapter 2**. CO₂ gasses with range of bulk isotopic compositions, heated to 1000°C for ≤4hrs as described in **chapter 2**, were also measured to monitor mass spectrometric non-linearity, however are not used in non-linearity correction. Data is then translated into the absolute reference frame (Dennis et al., 2011). Carbonate standards routinely measured to allow for translation are our Imperial Carrara Marble (ICM), ETH2 and ETH3 on Santa Maria and ETH1, ETH2 and ETH3 on Pinta with the IBEX. In addition, on the IBEX we also measure external standards ICM and ETH4 to monitor reproducibility of standards. Δ_{47} is corrected for acid fractionation by adding an acid fractionation factor 0.082‰ (Defliese et al., 2015). Masses 48 and 49 were used to detect the presence of contaminants. Δ_{48} is calculated as described in John and Bowen (2016). Samples with Δ_{48} offset values from heated gas lines >1.5‰ or 49 parameter values >0.2 were excluded from analysis following Davies and John (2017).

3) RESULTS

We use the measured stable oxygen isotopic values of outer prismatic layer carbonate ($\delta^{18}\text{O}_c$) (Table 2) in nautilus and sepia shells in combination with the isotope composition of seawater in which individuals grew ($\delta^{18}\text{O}_{sw}$) (Table 1) to calculate the oxygen isotope fractionation factor (α) between cephalopod aragonite and seawater. The measured values of $1000\ln\alpha$ for both the nautilus and sepia are higher than the inorganic precipitation experiments of Kim et al. (2007) taken to represent an approximation of precipitation under equilibrium conditions (Figure 5.2a). This corresponds to between a 2.5-6°C underestimate of growth temperature in nautilus samples and a 0.75-2°C underestimate of growth temperature in sepia (Figure 5.2b). The temperature dependence of α in sepia approximately mirrors that of inorganic precipitation experiments however the less well-defined correlation between $1000\ln\alpha$ and temperature ($R^2 = 0.64$, $p < 0.001$) in nautili is much steeper than that of near equilibrium precipitation.

The Δ_{47} values of 8 nautili samples measured fall below the combined inorganic Δ_{47} -temperature calibration of Davies and John (2018) assumed to approximate equilibrium precipitation. The remaining sample falls on the inorganic calibration line. The offset in nautili Δ_{47} is on average 0.031‰, ranging up to 0.082‰ (Figure 5.3a). This corresponds to 10°C average and up to 29°C overestimate in calculated shell formation temperature (Figure 5.3b). Choice of inorganic calibration line does not have a significant impact on this result. In contrast Δ_{47} values of sepia lie within error of the inorganic calibration of Davies and John

(2018) (Figure 3a). Notably the Δ_{47} -temperature dependence in sepiia closely reflects that of the inorganic calibration.

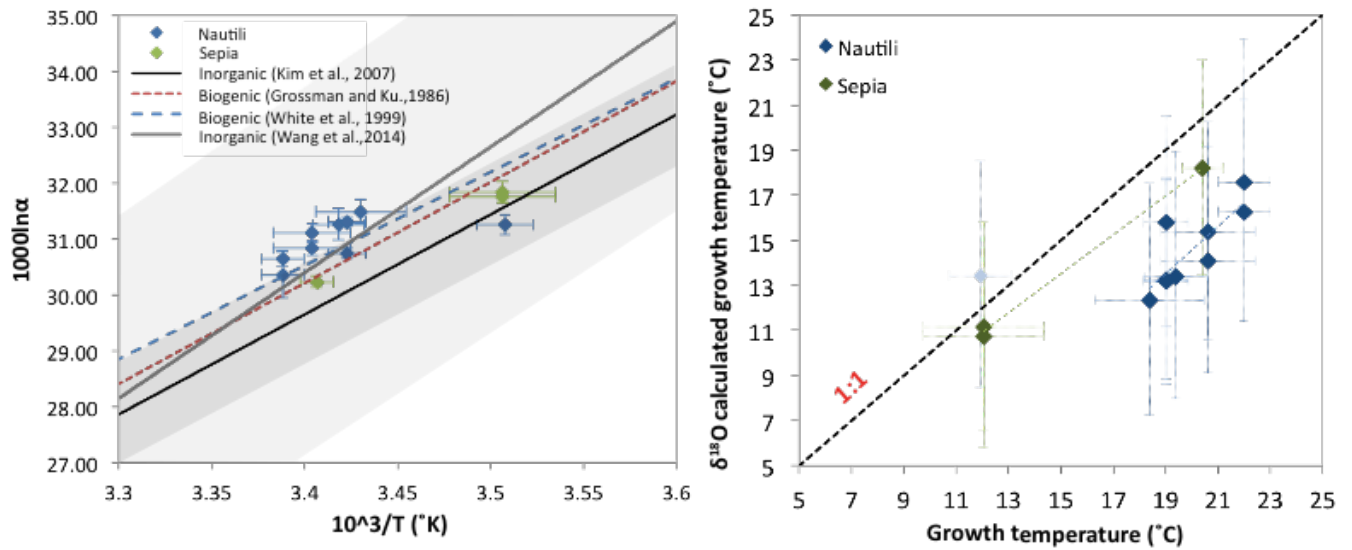


Figure 5.2 a) Temperature dependant oxygen isotope fractionation of inorganic precipitates (Kim et al. 2007; Wang et al. 2014), molluscs (Grossman and Ku, 1986; White et al. 2011) nautili and sepiia (this study) b) comparison between $\delta^{18}O$ calculated growth temperature (Kim *et al.*, 2007) and measured growth temperatures averaged to account for seasonal and temperature depth habitat variation (Locarnini *et al.* 2013) (Appendix Table 1a).

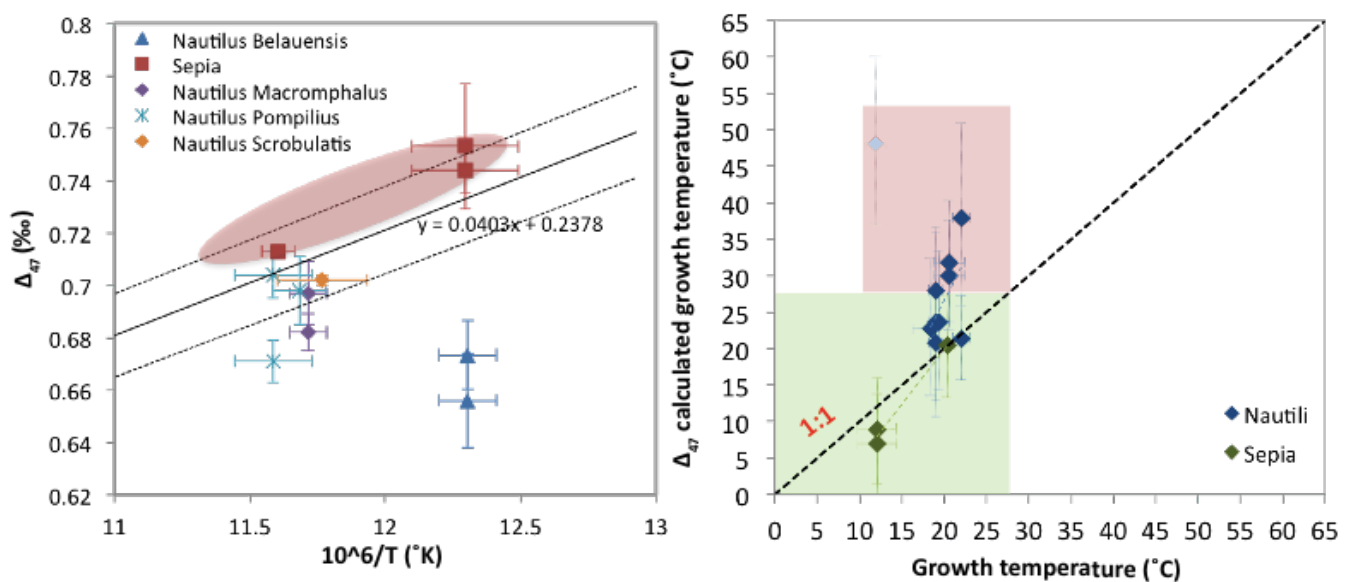


Figure 5.3 a) clumped isotope (Δ_{47}) temperature calibration of the nautili and sepiia compared with the combined inorganic calibration of Davies and John (2018) and b) comparison between Δ_{47} calculated growth temperature (Davies and John 2018) and measured growth temperatures averaged to account for seasonal and temperature depth habitat variation (Locarnini *et al.* 2013) (Appendix Table 1a). The green box highlights reasonable Δ_{47} calculated growth temperatures, the red box unreasonable Δ_{47} calculated growth temperatures.

Table 2. Sample stable and clumped isotope composition

Sample Name	Sample	n	$\delta^{18}\text{O}$ VPDB (Final) (‰)	SE (‰)	$\delta^{13}\text{C}$ VPDB (Final) (‰)	SE (‰)	Δ_{47} CDES (Final) (‰)	SE (‰)
ANNCO	Outer shell	3	0.85	0.02	0.75	0.08	0.63	0.01
ANPUO	Outer shell	4	0.92	0.15	0.34	0.06	0.65	0.02
ANWA	Outer shell	2	-0.74	0.40	-2.28	0.17	0.70	0.07
NFI	Outer shell	3	0.98	0.14	1.94	0.22	0.70	0.00
NLI	Outer shell	3	0.80	0.24	1.59	0.03	0.70	0.01
NNCL	Outer shell	3	0.32	0.05	2.08	0.04	0.70	0.01
NNCS	Outer shell	3	0.88	0.05	1.48	0.02	0.68	0.01
NTB1	Outer shell	3	0.47	0.05	1.74	0.30	0.70	0.01
NTB2	Outer shell	3	0.75	0.09	1.22	0.04	0.67	0.01
WNPUO1	Outer shell	3	0.33	0.11	1.79	0.03	0.68	0.01
SO1	Dorsal	4	1.16	0.18	-2.89	0.06	0.74	0.01
SO2	Dorsal	3	1.07	0.05	-2.89	0.11	0.75	0.01
SO3	Dorsal	3	0.03	0.05	-3.51	0.16	0.71	0.01
WNPUS1	Septum	3	0.66	0.10	1.30	0.06	0.67	0.00
WNPUS5	Septum	3	1.19	0.12	-0.97	0.09	0.70	0.01
WNPUS9	Septum	4	0.87	0.08	0.42	0.13	0.72	0.02
WNPUS13	Septum	4	1.23	0.04	-0.33	0.29	0.70	0.02
WNPUS17	Septum	5	1.24	0.09	-0.71	0.18	0.74	0.02
WNPUS21	Septum	3	1.26	0.07	-0.89	0.04	0.70	0.01
WNPUS25	Septum	4	0.74	0.31	-0.37	0.25	0.71	0.03
ANNCS1	Septum	4	0.94	0.21	1.32	0.17	0.67	0.00
ANNCS3	Septum	5	0.94	0.17	1.70	0.04	0.66	0.01
ANNCS5	Septum	5	1.20	0.15	1.33	0.03	0.69	0.01
ANNCS7	Septum	3	1.30	0.14	1.87	0.04	0.69	0.02
ANNCS10	Septum	4	0.73	0.17	0.51	0.09	0.67	0.01
ANNCS12	Septum	3	1.47	0.07	0.81	0.02	0.72	0.00
ANNCS15	Septum	3	1.45	0.13	0.66	0.03	0.71	0.01
ANNCS17	Septum	3	1.34	0.11	1.53	0.22	0.73	0.01
ANPUS1	Septum	3	0.91	0.16	1.03	0.05	0.68	0.01
ANPUS5	Septum	3	1.11	0.18	0.84	0.03	0.68	0.00
ANPUS10	Septum	5	1.42	0.04	1.21	0.16	0.70	0.01
ANPUS15	Septum	3	1.30	0.12	1.16	0.21	0.72	0.01

n is number of replicates

We present the $\delta^{18}\text{O}$, and Δ_{47} of selected septa from juvenile to most recent (Figure 5.4). Overall a gradual decrease in $\delta^{18}\text{O}$ is observed from post-embryonic (septa<23) to most recent septa. In individuals ANNCS and ANPUS there is no relationship between $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$, $\delta^{13}\text{C}$ varying non-predictably in consecutive septa. Septal $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ co-vary in WNPUS, $\delta^{18}\text{O}$ decreasing with from juvenile to more mature, this correlating with an increase in $\delta^{13}\text{C}$ (Figure 5.5).

All individuals show a strong correlation between decreasing Δ_{47} from juvenile to mature septa, ANPUS ($R^2 = 0.864$ $P < 0.0001$), ANNCS ($R^2 = 0.771$ $P < 0.0001$) and WNPUS ($R^2 = 0.798$ $P < 0.0001$) (Figure 5.4). In individuals ANPUS and ANNCS it was not possible to sample embryonic septa. In sample WNPUS the youngest septa (septa 23 to 25) are embryonic septa and exhibit higher Δ_{47} values corresponding to growth temperatures between 19.5°C to 23 °C (Figure 5.6), within error of known successful hatching temperatures (Landman et al., 1994).

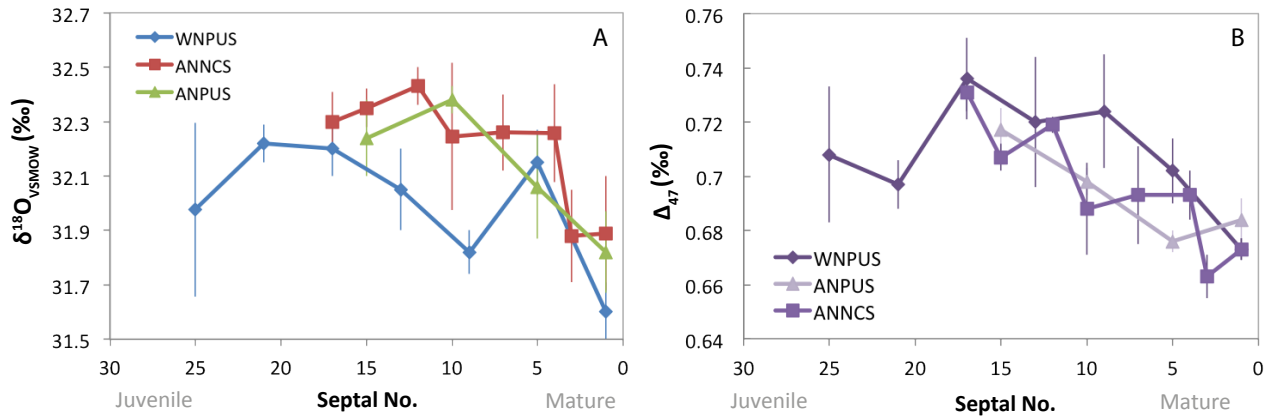


Figure 5.4 Change in a) $\delta^{18}\text{O}$ and b) Δ_{47} with increasing septal number from juvenile to most recently formed septa

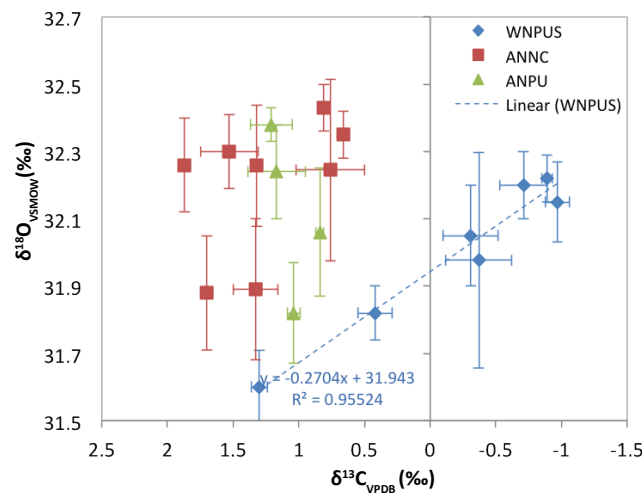


Figure 5.5 Relationships between septal $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ in *Nautilus Belauensis* and *Nautilus Macromphalus*

Between septa 12 and 17 calculated growth temperature based on $\delta^{18}\text{O}$ and Δ_{47} lie within error of actual temperatures, but start diverging in more mature septa (Figure 5.6). Δ_{47} values within this septal range also most accurately correspond with the range of potential growth temperatures given vertical migration of individuals within the water column and known growth temperatures on capture in the case of sample WNPUS. As with Δ_{47} measurements of outer shell aragonite, Δ_{47} derived temperatures for septa 3 to the most recent septa 1 of all three individuals are $>25^\circ\text{C}$.

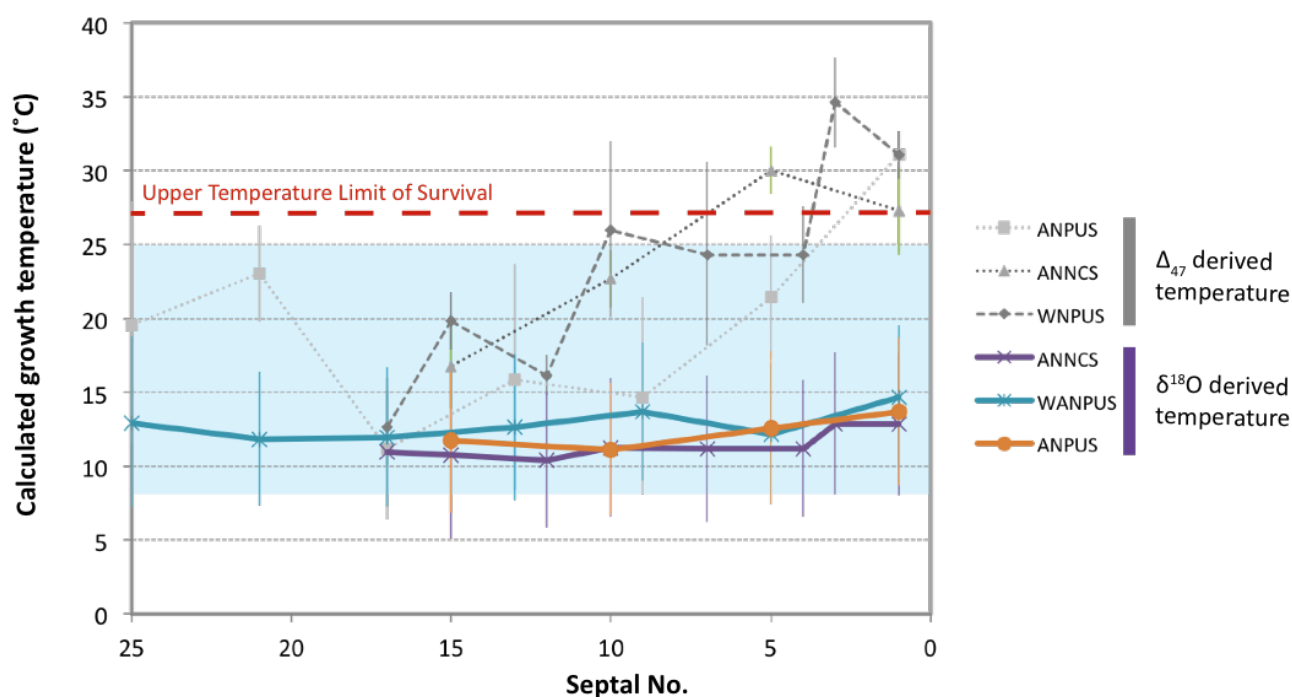


Figure 5.6 Calculated growth temperatures based on Δ_{47} (grayscale data points) and $\delta^{18}\text{O}$ coloured data points from juvenile to most recently formed Septa

4) DISCUSSION

4.1 The $\delta^{18}\text{O}$ composition of measured cephalopods

The assessment of equilibrium precipitation in oxygen isotopic composition is complex. This stems from uncertainty in the “true” $\delta^{18}\text{O}$ -temperature relationship at equilibrium. Two approaches exist for establishing $\delta^{18}\text{O}$ -temperature relationships; laboratory based empirical calibration using synthetically precipitated carbonates and field calibration, achieved by the measurement of natural samples with well-constrained growth temperatures. We compare our cephalopod data both with the inorganic aragonite calibrations of Kim et al. (2007) and Wang et al. (2013) and field calibrations of aragonitic molluscs by Grossman and Ku (1986) and White et al. (1999) (Figure 1).

The calculated temperature dependent $\delta^{18}\text{O}_{\text{aragonite-water}}$ fractionation of the cephalopods lies within error of inorganically determined $\delta^{18}\text{O}_{\text{aragonite-water}}$ fractionation of Kim et al. (2007) but more closely approximates inorganic $\delta^{18}\text{O}_{\text{aragonite-water}}$ fractionation determined by Wang et al (2013). The temperature dependence of fractionation determined by Wang et al (2013) is however much steeper than that demonstrated by Kim et al. (2007) and by biogenic calibrations (Grossman and Ku, 1986; White et al., 1999), and that of our nautili measurements.

“Vital effects” are defined as an offset in the isotopic composition of biogenic carbonates from precipitation in isotopic equilibrium with seawater. Group specific biogenic calibrations such as those of Grossman and Ku (1986) and White et al. (1999) account for potential vital effects in $\delta^{18}\text{O}$ -temperature relationship. It must be noted that despite the conclusion that all biogenic aragonite-water fractionation curves are statistically indistinguishable from inorganic aragonite-water fractionation, regardless of “vital effects”, (Kim et al., 2007) most researchers use the temperature dependent fractionation of aragonite molluscs of Grossman and Ku (1989) (Landman et al., 1994; Ohno et al., 2014) or ad-hoc calibrations (Oba et al. 1992) in preference to inorganic $\delta^{18}\text{O}$ -temperature calibrations. This is because biogenic calibrations such as Grossman and Ku 1989 produce more accurate growth temperature estimates for aquarium-reared specimens, with known growth temperatures, than the inorganic calibration of Kim et al. (2007) (Landman et al., 1994).

Although many modern biogenic aragonites are precipitated *at or very near* oxygen isotope equilibrium with ambient water (Kim et al., 2007) there is nevertheless a positive offset between biogenic calibrations and inorganic aragonite-water fractionation of Kim et al., (2007). Whether or not this reflects analytical error, a departure from equilibrium precipitation in inorganic calibrations or a true “vital effect” in $\delta^{18}\text{O}$ however remains uncertain. Ohno et al. (2014) demonstrate that $\delta^{18}\text{O}$ of outer shell walls more reasonably reflect seawater temperature based on the calibration of Grossman and Ku (1986) than septal aragonite grown at the same time. The discovery of inter-skeletal variability in $\delta^{18}\text{O}$ of nautilus aragonite supports hypothesis for non-equilibrium precipitation in the $\delta^{18}\text{O}$ of the nautili.

The $\delta^{18}\text{O}_{\text{aragonite-water}}$ fractionation vs. temperature calibration of the sepia (cuttlefish) differs slightly from that of the nautili and is closely correlating with inorganic $\delta^{18}\text{O}_{\text{aragonite-water}}$ fractionation (Kim et al., 2007).

Linearly correlated depletions in the $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ compositions of biogenic carbonate are considered to be the result of kinetic effects during the hydration and hydroxylation of CO_2 (McConnaughey, 1989a; McConnaughey, 1989b) calculated to be the rate-limiting step in inorganic carbon speciation (Johnson, 1982). We observe no trend in $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ in septal aragonite in specimens ANPU and ANNCS but strong positive correlation in WNPUS ($R^2 =$

0.955 $p < 0.001$) (Figure 5.5). This reflects the patterns observed in corals (Saenger et al., 2012) however unlike surface and deep sea corals, urchins and encrusting algae (McConnaughey, 1989b), Nautili are known to exhibit significant vertical migration in the water column, the extent of which is still not fully understood (Auclair et al., 2004; Zakharov et al., 2006; Dunstan, Ward and Marshall, 2011a; Dunstan, Ward and Marshall, 2011b). The $\delta^{13}\text{C}$ value of seawater dissolved inorganic carbonate (DIC) and temperature both decrease with depth; thus correlation between $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ of septal carbonate could be due to a change in depth habitat over the lifespan of the nautilus. Data from Waikiki aquarium specimens also support septal variation in $\delta^{13}\text{C}$ reflecting changes in $\delta^{13}\text{C}$ DIC due to the decomposition and sinking of organic matter and dissolution of CaCO_3 (Landman et al., 1994). This is not to say that the $\delta^{13}\text{C}$ of shell carbonate is not influenced by vital effects; indeed the $\delta^{13}\text{C}$ values of mollusk shells has been shown to represent the $\delta^{13}\text{C}$ of seawater DIC, the $\delta^{13}\text{C}$ of respired CO_2 and metabolic $\delta^{13}\text{C}$ (McConnaughey, 1989a; Auclair et al., 2004; McConnaughey and Gillikin, 2008; Beirne et al., 2012).

4.2 Δ_{47} of measured cephalopods

A substantial challenge facing carbonate clumped isotope thermometry is the potential for kinetic or metabolic processes resulting in disequilibrium Δ_{47} signatures. Dennis et al. (2013) illustrate a negative offset in modern nautili from the Ghosh et al. (2006) inorganic calibration. Recent improvements in data processing for clumped isotope analysis (Daëron et al., 2016; Schauer et al., 2016) and comprehensive inorganic calibration of the thermometer over a wide temperature range (Kluge et al., 2015; Bonifacie et al., 2016; Kelson et al., 2017) have reduced uncertainties in the inorganic Δ_{47} -temperature relationship enabling non-equilibrium Δ_{47} signals to be more reliably identified. We plot the clumped isotopic composition of outer shell aragonite in nautili and sepia, reprocessed in the Santrock data correction parameters for compatibility alongside that of Dennis et al. (2013) (Figure 5.7) and note remarkable congruity of both nautili datasets. While we observe clear non-equilibrium effects in the nautili this is not the case for sepia. Sepia measurements of this study much more closely align with the inorganic Δ_{47} relationship however this is not reflected in the measurements of Dennis et al. (2013).

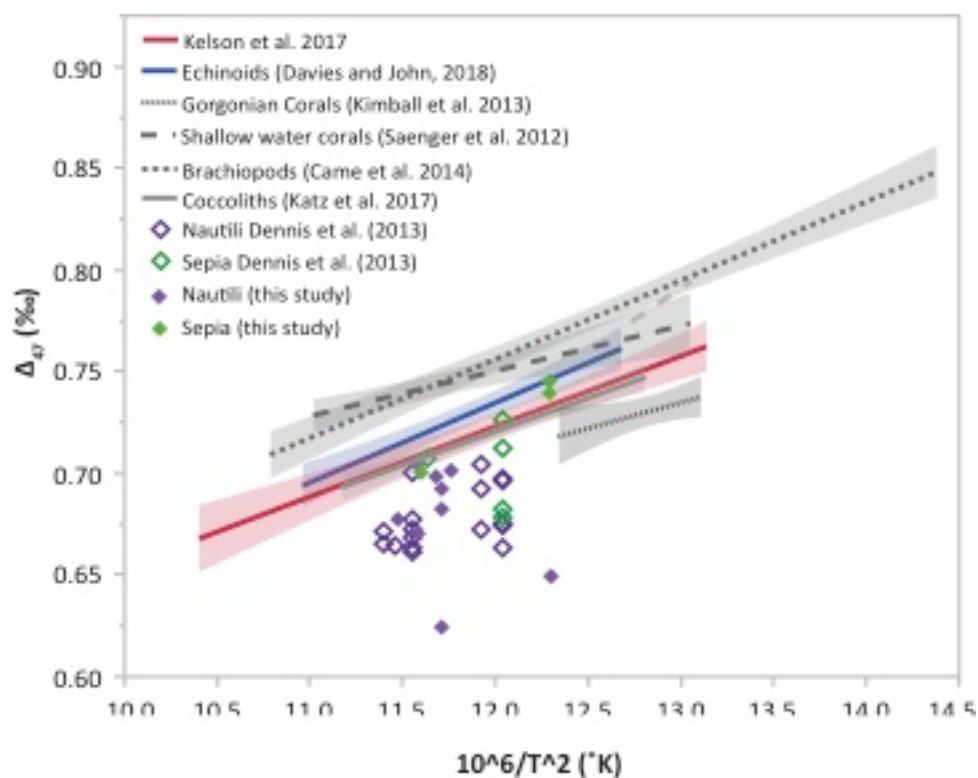


Figure 5.7 Correlation between growth temperature and Δ_{47} in nautili and sepia compared with the combined inorganic calibration of Davies and John (2018) echinoids (Davies and John 2018), brachiopods (Came *et al.* 2014) shallow water corals (Saenger *et al.* 2012) and gorgonian corals (Kimball *et al.* 2015). Δ_{47} CDES values are presented in the 25°C reference frame adjusting the acid fractionation factor to a common 0.082‰ (Defliese *et al.* 2015) using the Santrock *et al.* 1985 ^{17}O correction parameters. Shaded errors reflect 95% confidence limits on regression.

We compare Δ_{47} of cephalopod data, with other biogenic and non-biogenic carbonates (Figures 5.7). To allow accurate comparison all values are corrected using Santrock ^{17}O correction parameters and corrected for acid digestion by projecting the data at 25°C using an AFF 0.082‰ (Defliese *et al.*, 2015). Whilst most biogenic calibrations are positively offset from the inorganic Δ_{47} -temperature calibration, only the gorgonian corals (Kimball *et al.*, 2013) and cephalopods (Dennis *et al.* 2018, this study) display a negative offset. This offset is similar, although of smaller magnitude, to that observed in speleothems (Daëron *et al.*, 2011; Kluge and Affek, 2012; Affek and Zaarur, 2014; Affek *et al.*, 2014).

To verify disequilibrium precipitation in Δ_{47} we plot cephalopod Δ_{47} -growth temperature against growth temperature based on $\delta^{18}\text{O}$ measurements (Figure 5.3b). The Δ_{47} and $\delta^{18}\text{O}$ of sepia fall on the 1:1 line while calculated growth temperature based on Nautili Δ_{47} is much higher. Indeed 33% of *Nautilus* outer shell and all *Nautilus* peristome measurements produce temperatures above the threshold at which nautili can survive (Martin *et al.* 1978, Saunders, 1984).

In previous studies differences in observed Δ_{47} -temperature calibration in biological carbonates and inorganic precipitates have been putatively linked to uncertainties in inter-laboratory calibration of the clumped isotope thermometer (Dennis et al., 2013; Henkes et al., 2013). We circumvent this problem by comparing cephalopod values measured at Imperial College London to our “in house” inorganic calibration (see Davies and John, 2018). While nautili measurements from this study are negatively offset from the inorganic calibration, echinoids measured during the same measurement period (Davies and John, 2018) exhibit a positive offset (Figure 5.7).

Septal $\delta^{18}\text{O}$ measurements follow a well-documented trend (Coheran *et al.* 1989, Landman *et al.* 1983, Taylor and Ward 1983, Oba *et al.* 1992, Zakharov et al. 2006), decreasing with increased septal number from most recently formed to juvenile septa. This pattern is reflected in septal Δ_{47} values, which also follow a general decreasing trend with increasing septal number with the exception of embryonic septa 4-7 and septum 11. The co-varying decrease in both septal $\delta^{18}\text{O}$ and Δ_{47} is not unexpected as both are, under equilibrium conditions, a function of growth temperature. Increase in temperature with decrease in septal number is much more significant in Δ_{47} derived temperatures than $\delta^{18}\text{O}$ derived temperatures (Figure 5.6) If the decrease in both $\delta^{18}\text{O}$ and Δ_{47} with increasing maturity is a disequilibrium signal, it is a result of a process that affects clumped isotopes more than $\delta^{18}\text{O}$.

Δ_{47} values in most recently formed septa 27 and 31 in sample ANNCS, 22 and 26 in ANPUS and 31 in WNPUS reflect temperatures of 27.3-34°C. This is above both the measured sea surfaces temperature in New Caledonia and Pualu, 24.6 and 28.9°C respectively (Figure 5.6). *Nautilus Macromaphus* are known to stop eating at 25°C and die at 27°C (Martin *et al.* 1978, Saunders 1984). These measurements indicate non-equilibrium precipitation in Δ_{47} of both septal and outer shell aragonite the degree of disequilibrium precipitation in septal aragonite increasing from septum 12 upwards. Δ_{47} calculated temperature in juvenile septa is within the range of estimated growth temperature based on temperature depth profiles and capture records and $\delta^{18}\text{O}$. The Δ_{47} values of embryonic septa in sample WNPUS surprisingly reflect reasonable temperatures in line with hatching temperatures of 22°C at Kanazi and Waiki aquariums.

4.3 Cephalopod bio-mineralization and isotopic effects

The shells of both nautili and sepia are deposited as aragonite in two layers, an inner nacreous layer and an outer prismatic layer. Theoretical calculations (Schauble et al. 2006) indicate that chemistry should have little effect on estimated equilibrium constants for the formation of $^{13}\text{C}^{18}\text{O}^{16}\text{O}^{2-}$. The theoretically predicted difference in Δ_{47} aragonite and Δ_{47} calcite as a result of isotopic fraction during phosphoric digestion at 25°C is 0.002‰ with aragonite predicted Δ_{47} lower than that of calcite. This offset is in the same direction but much smaller in magnitude than the Δ_{47} offset of nautili from our combined inorganic calibration.

The greatest Δ_{47} offset relative to the inorganic calibration of Davies and John (2018) is observed in aragonite taken from the outer prismatic layer of shells. The clumped isotope sample size requirement necessitated averaging several growth bands. Linzmeier et al. (2016) report the $\delta^{18}\text{O}$ differences in grow bands below the variation that would result in a significant offset in Δ_{47} as a result of mixing of two end members with varying $\delta^{18}\text{O}$ (Lohmann, 2015).

In current models, shell formation in cephalopod molluscs is thought to occur primarily via extrapallial fluid (EPF) secreted by the outer mantle epithelium (OME) (Figure 5.8). The constituents required for biomineralization are provided by the haemolymph, a transport fluid analogous to blood in molluscs. Ca^{2+} , is thought to be transported through cell walls towards the EPF by active transport via the enzyme Ca-ATPase while protons (H^+) are may either be actively removed of the from the EPF by trans-membrane proton pumps such as H^+ -ATPase (Coimbra et al. 1988) or removed by reacting with the bicarbonate ion to produce CO_2 and water (Wilbur and Saleuddin, 1983), catalysed by carbonic anhydrase. Inorganic ions such as Na^+ , Mg^{2+} , K^+ Cl^- and SO_4^{2-} have been putatively identified in the EPF, impacting the overall ion activity of solution. H_2O and $\text{CO}_{2(\text{aq})}$ diffuse through the epithelial membrane into the EPF where they react to produce HCO_3^- and CO_3^{2-} .

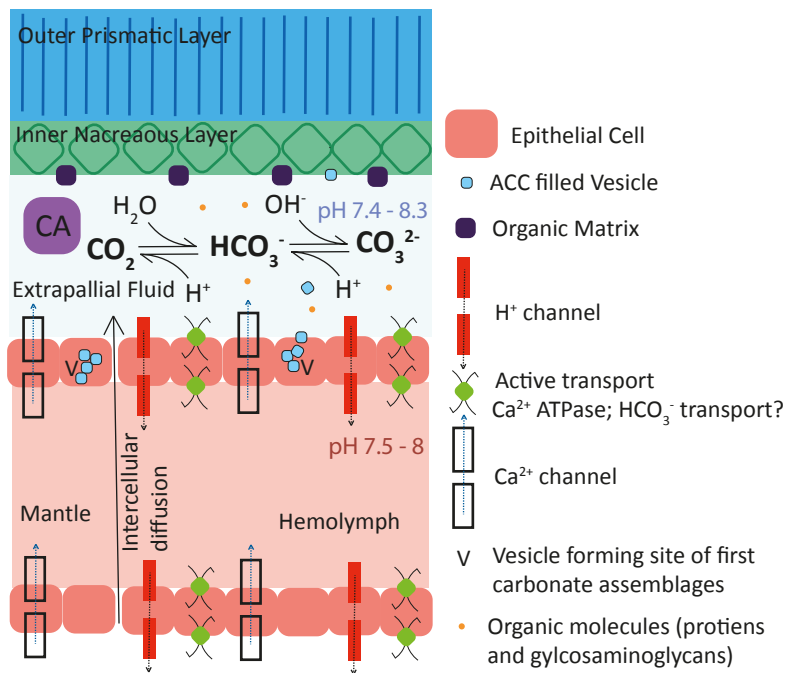


Figure 5.8 Model of biomineralization in molluscs Modified from Carre et al. (2006) in Immenhauser et al. 2016.

The conventional hypothesis (Urey, Epstein) of aragonite shell precipitation in oxygen isotope equilibrium with surrounding seawater is maintained by prepositions that (1) the main source of oxygen in CaCO₃ is H₂O (2) body fluids are constantly replenished by ambient water and (3) water can pass freely through epithelium to reach the extrapallial space or can be incorporated into amorphous (ACC) vesicles. The current model of biomineralization however comprises steps that may incorporate effects resulting in non-equilibrium precipitation in Δ_{47} and/or $\delta^{18}\text{O}$. These steps include diffusion of CO₂ across epithelial membranes, chemical kinetics of the hydration and hydroxylation of aqueous CO₂ and kinetic effects during the adsorption of HCO₃⁻ onto surface layers of growing grains, pH and salinity effects (Figure 5.9).

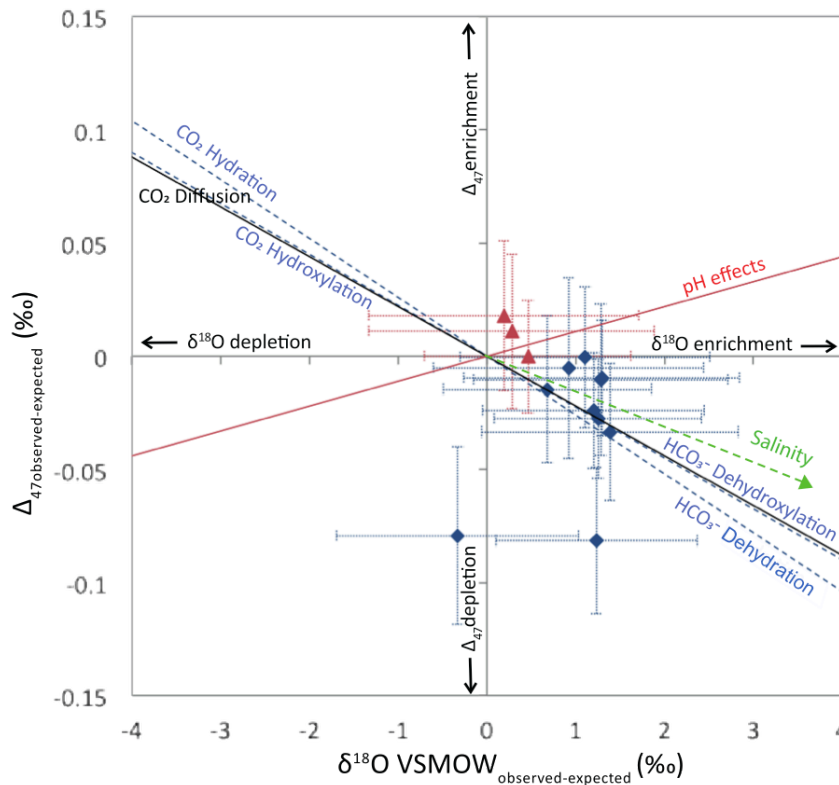


Figure 5.9 Correlation between fractionation in $\delta^{18}\text{O}$ and Δ_{47} in cephalopods.

Vector trajectories for effects of different processes on stable isotope signatures such as pH effects (Tripathi et al. 2015), salinity (Hill *et al.* 2014), CO_2 diffusion (Thiagarajan et al. 2011), CO_2 hydration and hydroxylation reactions (Guo, 2008). Y-axis error bars represent propagated error on calculated expected Δ_{47} based on calibration error, error in growth temperature and standard error on Δ_{47} observed. X-axis error bars represent propagated error on expected $\delta^{18}\text{O}$ based on error in $\delta^{18}\text{O}_{\text{sw}}$ at growth location, error on $\delta^{18}\text{O}$ calibration and observed $\delta^{18}\text{O}$ measured at Imperial College London.

The fluid composition of isolated environments such as calcification sites in cephalopods has the potential to yield significant deviation in salinity from seawater. Nautili, living below depths of 240 m, are thought to excrete cameral liquid from chambers by actively transporting Na^+ and Cl^- across the siphuncular epithelium creating an osmotic gradient, which produces a passive movement of cameral liquid across the siphuncle into the blood (Greenwald et al. 1980). This is evidence for the capacity of active transport of Na^+ and Cl^- across cell membranes in nautili permitting the control of salinity in intercellular fluids (including the EPF). Shallow water sepia in contrast maintain buoyancy by reducing osmotic pressure of the cameral liquid allowing osmosis out of the chamber (Denton et al 1961). The salinity of cameral fluid in nautilus chambers may have important implications for septal Δ_{47} as septal carbonate is a combination of biogenic aragonite and inorganic carbonate precipitated directly from chamber fluid (Figure 5.9).

The cameral liquid and EPF in cephalopod molluscs contains of carbonic acid (H_2CO_3), dissolved carbon dioxide ($\text{CO}_{2(\text{aq})}$), bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) ions. Together

these are referred to as dissolved inorganic carbonate (DIC). Theoretical modelling by Hill et al. (2014) indicates each species has a distinct equilibrium clumped isotope signature, $\Delta_{47}(\text{H}_2\text{CO}_3) > \Delta_{47}(\text{HCO}_3^-) > \Delta_{47}(\text{equilibrium calcite}) > \Delta_{47}(\text{CO}_3^{2-})$, thus carbonate speciation within the parent fluid (EPF) has the potential to affect clumped isotope composition of precipitating carbonate minerals (Hill et al., 2014). The difference between $\Delta_{47}(\text{HCO}_3^-)$ and $\Delta_{47}(\text{CO}_3^{2-})$ at 25°C is predicted to be 0.018‰ (Guo et al., 2008) to >0.033‰ (Hill et al., 2014). Based on the empirical formulas of Millero et al. (2006) theoretical calculations indicate increases in salinity decrease the maximum abundance of the HCO_3^- impacting the Δ_{63} of the DIC pool (Figure 5.10) (Hill et al. 2014). Increasing salinity of the EPF thus has a positive impact on the aragonite saturation state within the fluid and would be of a biological advantage to the organism. Furthermore increasing salinity shifts the crossover of Δ_{63} DIC with equilibrium carbonate to lower pH values such that if precipitation pH is close to crossover pH, changes in salinity may have produce a Δ_{63} DIC value 0.024‰ lower than equilibrium Δ_{63} DIC (Hill et al. 2014) (Figure 5.10). It is however worth noting that for fluid salinities <50 PSU the crossover pH (~8.7-10) is above measured pH of the EPF (7.4-8.3) (Hahn et al., 2012; Heinemann et al., 2012) thus invoking salinity driven vital effects in Δ_{47} would require calcifying fluid salinities >50 PSU.

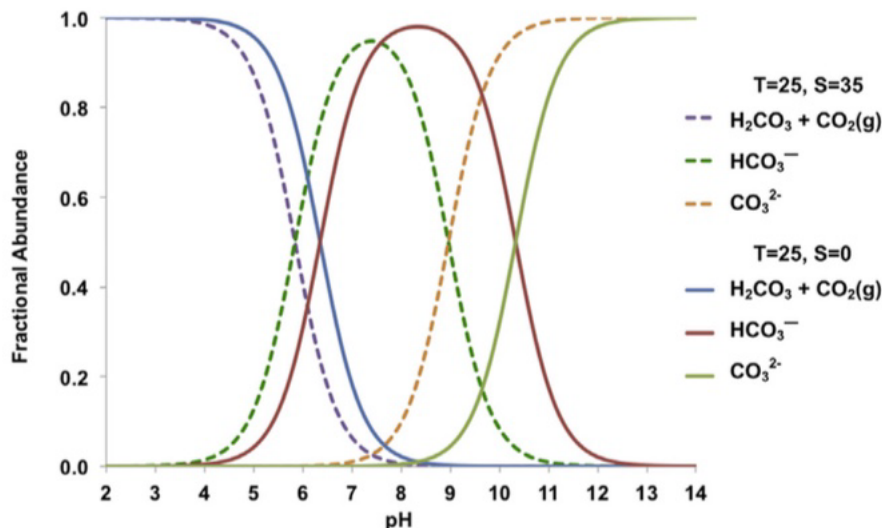


Figure 5.10 Effect of salinity (S) on the relative abundances of DIC species v.s pH (Hill et al. 2014)

The above discussion however must be caveated by reference to empirical data of Kluge and John (2015) in which addition of NaCl up to 375 PSU produce no effect on Δ_{47} values at mineral formation temperatures 20 to 100°C. Variation in Δ_{47} high salinity - Δ_{47} low salinity of up to ~0.055‰ is however observed on increasing CaCl_2 concentration of the parent fluid. Kluge and John (2018) link this observation to Ca^{2+} concentration in solution, offsets in Δ_{47} between

saline and non saline solution being observed at high Ca^{2+} concentrations, as is likely to exist in calcifying fluids.

Kinetic effects during biomineralization have been implicated in previous studies. The hydration and/or hydroxylation of aqueous CO_2 is considered to be a factor in enrichment in Δ_{47} and associated depletion in $\delta^{18}\text{O}$ in surface corals (Saenger et al., 2012). Similarly kinetic effects during hydration of aqueous CO_2 within the calcifying fluid of modern brachiopods have been linked to depletion in $\delta^{18}\text{O}$ and enrichment in Δ_{47} (Banjai et al. 2018). This reflects the initial findings of (McConnaughey, 1989a; McConnaughey, 1989b) in which linear correlations between $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ in coral, foraminifers, algae and cicadoid urchins are associated with kinetic effects during CO_2 hydration and hydroxylation. In contrast, in the nautili we observe a considerable depletion in shell Δ_{47} and associated enrichment in $\delta^{18}\text{O}$ in comparison with approximated equilibrium precipitation (Kim et al. 2007, Davies and John 2018) (Figure 5.9).

In this respect the nautili appear to have more in common with speleothems, in which CO_2 degassing results in enriched $\delta^{18}\text{O}$ and Δ_{47} , than with surface corals and brachiopods. In speleothems disequilibrium effects have been demonstrated to occur as a result of bicarbonate dehydration (Eqn 5.2) and dehydroxylation (Eqn 5.3) (Daeron et al., 2011; Kluge and Affek, 2012; Affek et al. 2014).



The link between kinetic effects during dehydration/dehydroxylation in speleothems and biological carbonates such as the nautili, may be expanded on by considering the current model for biomineralization in cephalopods (Figure 5.8) and its associated uncertainties. These uncertainties are as follows (1) whether the $\text{CO}_2/\text{H}_2\text{CO}_3^-$ conversion occurs in the cytoplasm of outer epithelial cells, connective tissues or in the EPF (2) lack of demonstration of the involvement of active Ca-ATPases and in particular H-ATPases for reabsorbing protons (Wilbur et al. 1983, Coimbra et al. 1988) (3) ambiguity as to the location of the carbonic anhydrase (CA), recent findings indicating mantle specific CA as observed in gastropods (Le Roy et al., 2012) and CA both secreted and incorporated into the shell matrix as in freshwater bivalves (Marie et al. 2010). Focusing first on (2) if H-ATPases are not present to absorb protons from the EPF, the most likely pathway for increasing pH and maintaining

electroneutrality of the fluid on the addition of Ca^{2+} to solution is dehydration of HCO_3^- (Eqn 1) as proposed by (Wilbur and Saleuddin, 1983). Pertaining to (3) if carbonic anhydrase is not present at the site of dehydration, kinetic effects may produce a lower proportion of ^{13}C - ^{18}O substituted isotopologues in the residual HCO_3^- compared to that at expected equilibrium (Guo et al. 2007, 2009), a signature which is then inherited by carbonate precipitated from that HCO_3^- . Furthermore if (1) $\text{CO}_2/\text{HCO}_3^-$ conversion occurs in epithelial cells or within the mantle under the presence of carbonic anhydrase and is then delivered to the EPF as HCO_3^- by bicarbonate pumps, where dehydration occurs in the absence of CA this would result in amplification of ^{13}C - ^{18}O isotopic disequilibrium. Within the pH range of mantle and EPF dehydration is the dominant mechanism in HCO_3^- degassing (Johnson, 1982).

The process of biomineral formation at the fluid-mineral interface in cephalopods remains poorly understood. There is evidence for the presence of organic molecules within the EPF and at sites of mineral nucleation. Some molecules appear to inhibit and others promote mineral formation (Nudelman et al., 2006). Both an initial gel silk phase, usually the major protein fraction including acidic proteins, and an ordered structural chitin, a long chain insoluble polymer on which the first formed mineral (ACC) forms are considered to be important in bio mineralization (Addadi et al., 2006).

Sancho-Tomas et al. (2014) recently demonstrated the role of the soluble organic matrix for the formation of nacre through ACC precursor phases for *Nautilus pompilius*. An experimental study by Wang et al. (2009) indicates that macromolecules including carboxylated (acidic) proteins also control the Mg content of ACC in vitro. It is reasonable therefore to assume that electrostatic topology of the organic mineralization matrix in mollusks could have an influence on the adsorption of CO_3^{2-} and/or HCO_3^- isotopologues onto the aragonite surface thus impacting Δ_{47} . However these effects are yet to be empirically investigated.

The concept of an amorphous precursor phase with different thermodynamic and crystallographic properties, formed within the isolated environment of vesicles is important when considering non-equilibrium signatures in $\delta^{18}\text{O}$ and Δ_{47} of mollusk carbonate. Vesicles, thought to consist primarily of ACC, have been identified in various mollusc tissues, including the hemolymph and epithelial cells (Watabe et al. 1976, Neff et al. 1972). It is hypothesized that these vesicles, particularly small granules in epithelial cells, may transport ACC to the shell interface. The mechanism by which ACC is converted into crystalline carbonate remains

uncertain (Addadi et al., 2003). If ACC is converted to aragonite forming nacre by dissolution and re-precipitation in interaction with H₂O in the EPF then the unique Δ_{47} composition of ACC will be lost. If however this conversion occurs under closed conditions or without breaking of C-O bonds, preservation of a unique non-environmentally representative Δ_{47} signal may still occur (Davies and John 2018). As the process of nucleation and growth of the crystalline on the organic matrix at the expense of ACC remains uncertain, especially with regard to the true function of the EPF (Simkiss et al. 1989, Addadi et al. 2006), the inheritance of the Δ_{47} signature of the ACC remains a topic for future investigation, beyond the reach of this study.

4.4 Implications for insight into ontogenesis and growth habitat deduced by $\delta^{18}\text{O}$ reconstructions

The $\delta^{18}\text{O}$ composition of modern cephalopod calcite, particularly in the Nautili, has been widely used for reconstruction of growth habitat over the lifespan of specimens investigated (Landman et al. 1983, Coheran et al. 1989, Taylor and Ward 1983, Oba et al. 1992, Auclair et al. 2006, Zacharov et al. 2006) with varying degrees of success. Most studies (Landman et al.; Auclair et al. 2006, Zacharov et al. 2006) refer to the temperature dependent fractionation of aragonite molluscs of Grossman and Ku (1989). This produces reasonable temperature estimates based on the $\delta^{18}\text{O}$ of aquarium-reared specimens. Non-equilibrium fractionation in $\delta^{18}\text{O}$ of molluscs, contrary to the common model of Epstein et al. (1951), has been previously discussed (White et al. 2006). If the assumption is made that inorganic calibrations best represent equilibrium fractionation between aragonite and water, researchers are recognising the existence of group specific vital effects in $\delta^{18}\text{O}$ in cephalopods, but accounting for them by the use of field calibration.

This however assumes that “vital effects” remain constant throughout the lifespan of the specimen. We have presented evidence that vital-effects in the Δ_{47} composition of septal aragonite are amplified with increased septal number from juvenile to mature specimens. The hydration/dehydration of CO₂ and by inference dehydration/dehydroxylation of HCO₃ has been demonstrated to occur at the same rate for Δ_{47} as $\delta^{18}\text{O}$ suggesting disequilibrium in one system will also manifest in the other (Affek, 2013). This therefore reduces the certainty with which $\delta^{18}\text{O}$ can be used to reconstruct temperature and, by inference depth habitat, of modern nautili and extinct groups such as the ammonites.

4.5 Implications for environmental reconstruction using the $\delta^{18}\text{O}$ and Δ_{47} of cephalopod aragonite

Ammonites and belemnites have long been targets for environmental reconstruction by stable isotope geochemistry (Stahl and Jordan, 1969; Wierzbowski, 2007; Dennis et al., 2013). The outer aragonitic shells of ammonites are inherently unstable, easily altering to calcite. Unaltered aragonitic cephalopod carbonate is however found as far back as the Lower Carboniferous (Hallam and O'Hara, 1962), with more common occurrences in the Mesozoic and Cenozoic. The aragonitic composition of ammonids makes the identification of specimens altered by diagenesis much more straightforward. The calcitic rostra of belemnites are more diagenetically stable and were the first fossils to be used for paleotemperature calculations by the oxygen isotope method (Epstein et al., 1951) and since then have been widely used in Mesozoic sea temperature reconstruction (Bowen, 1966; Stevens and Clayton, 1971; Podlaha et al., 1998; Korte et al., 2009; Price and Passey, 2013).

Ammonites form part of the ectochelate group, cephalopods with an external shell, the only living species of which are four nautili species from the Indo-Pacific region. Cladistic analysis and some paleontological data however suggests that ammonids are more closely related to modern coeloids (Jacobs and Landman 1933, Flower 1961, Engeser 1990;1996), including *Sepia*. The closest living relatives of belemnites are *Spirula spirula*, the sepiids, and the teuthid squids (Fuchs et al., 2012; Klug et al., 2016).

We show a significant deviation in the Δ_{47} composition of the Nautili in comparison with inorganically precipitated carbonate indicating strong “vital effects” in clumped isotope composition. Samples from the dorsal regions of *Sepia* however widely conform to the inorganic Δ_{47} -temperature relationship (Figure 5.3). Δ_{47} offsets in modern nautili have also been tentatively identified in Late Cretaceous ammonoids *Baculites compressus* and *Hoploscaphites nebrascensis* from the Western Interior Seaway (Dennis et al. 2013) implying key similarities in biomineralization in these morphologically similar ectochelate groups. Interestingly although fossil ammonoids may be more closely related to coeloids such as *Sepia* their behaviour in terms of clumped isotope fractionation does not reflect this (Figures 5.2,3). The close to equilibrium Δ_{47} -temperature relationship of *Sepia* (Figure 5.3) however indicates that clumped isotope paleotemperature reconstruction using belemnites may be a better prospect.

With exception of Dennis et al. (2013) there have been no seawater temperature/ $\delta^{18}\text{O}$ reconstructions based on the clumped isotopic composition of cephalopods. Considering the substantial offset in nautilus Δ_{47} from inorganic calibrations, recent findings of Stevens et al. (2017) that the $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ of the belemnite rostra may indicate a combination of kinetic and metabolic vital effects, and evidence from this study that deviations in $\delta^{18}\text{O}$ from expected values may be amplified in Δ_{47} we urge caution in the indiscriminate application of the clumped isotope thermometer to cephalopod groups. Moreover the practice of adding a constant Δ_{47} offset to non-equilibrium Δ_{47} values of ammonoid aragonite (Dennis et al., 2013) may not be sufficient to account for non-equilibrium effects, as there is significant inter-skeletal variability within a single specimen. The Δ_{47} variability between juvenile and mature chambers can result in 15 to 22°C difference in calculated seawater temperature.

5) CONCLUSION

In conclusion we show a negative offset in nautilus aragonite of up to 0.08 (‰) corresponding to an overestimate in carbonate formation temperature of 22°C. In contrast sepia appear to precipitate *at or very near* oxygen and clumped isotope equilibrium. We propose the correlated negative offset in Δ_{47} and positive offset in $\delta^{18}\text{O}$, compared with expected values, may occur as a result of kinetic effects during the dehydration of HCO_3^- in the extrapallial fluid of nautili. The negative Δ_{47} offset in nautili is highly variable inter-skeletally within individual specimens ranging from no apparent offset to 0.08‰, and the offset increases from juvenile to most recently formed septa. The significant non-predictable offsets in nautilus Δ_{47} is a potential significant barrier in paleo-temperature and $\delta^{18}\text{O}_{\text{sw}}$ reconstruction using the genetically and morphologically related ammonids. We would therefore advise caution in the future use of Δ_{47} in paleoclimate reconstructions based on cephalopod groups.

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APPENDIX

Table 1A depth weighted average temperature calculation for the nautili and sepia

Depth (m)	Detections per hr total at depth (Dunstan et al., 2016)	Ratio of detections per hour at depth	Average Temperature at depth (°C) (Locarnini et al., 2013)					
			Palau	New Caledonia	Taema Bank	Lindeman Island	Florida Island	Osprey Reef
130-175	15.6	0.198726115	16.1	20.89	24.55	22.14	23.85	22.37
175-225	31.2	0.397452229	12.31	19.72	22.15	20.32	20.03	20.13
225-275	17.8	0.226751592	10.265	18.56	19.46	18.61	16.1	17.86
275-325	9.1	0.115923567	8.98	17.32	16.52	16.75	12.91	15.79
325-525	4.8	0.061146497	7.79	13.44	10.21	12.56	8.91	10.77
Weighted average growth temperature (°C)			11.93	19.02	20.63	19.4	18.39	18.98
Weighted standard deviation (°C)			2.7	1.92	4.02	2.65	4.63	3.24

	Average Temperature at depth (°C) (Locarnini et al., 2013)	
Depth	Littlehampton	Qalhat
0m	12.1	n/a
50m	11.98	22.52
200m	n/a	18.31
Growth Depth Average (°C)	12.04	20.415
Growth Depth SD (°C)	3.255	1.095

Table 2 depth weighted average $\delta^{18}\text{O}$ calculations for the nautili and sepia

Depth (m)	Detections per hr total at depth (Dunstan et al.,2016)	Ratio of detections per hour at depth	Average $\delta^{18}\text{O}$ at depth (%) (Le Grande and Schmidt., 2016)					
			Palau	New Caledonia	Taema Bank	Lindeman Island	Florida Island	Osprey Reef
130-175	15.6	0.198726115	0.13	0.54	0.72	0.52	0.54	0.52
175-225	31.2	0.397452229	0.06	0.53	0.67	0.53	0.52	0.5
225-275	17.8	0.226751592	0.03	0.51	0.52	0.49	0.39	0.44
275-325	9.1	0.115923567	0.03	0.46	0.35	0.43	0.26	0.36
325-525	4.8	0.061146497	0.03	0.32	0.13	0.31	0.11	0.21
Weighted average growth temperature (°C)			0.62	0.51	0.58	0.49	0.44	0.46
Weighted standard deviation (°C)			0.04	0.06	0.18	0.06	0.14	0.09

	Average $\delta^{18}\text{O}$ at depth (°C) (Le Grande and Schmidt., 2016)	
Depth	Littlehampton	Qalhat
0m	0.27	n/a
20m	0.29	n/a
50m	n/a	0.64
200m	n/a	0.79
Growth Depth Average (°C)	0.28	0.715
Growth Depth SD (°C)	0.01	0.075

Chapter 6: Thesis Outcomes and Conclusions

1) CONCLUSIONS

The carbonate clumped isotope thermometer is a useful tool in palaeoclimate reconstruction, especially in environments where the $\delta^{18}\text{O}$ of the water from which carbonate formation occurred is poorly constrained. Manifested advantages of carbonate clumped isotope thermometry initially went beyond this, congruency in the 0-40°C Δ_{47} -temperature calibration of both abiotic and biogenic materials (Eiler et al. 2011; Ghosh et al. 2006a, 2007; Came et al. 2007; Eagle et al. 2010; Tripathi et al. 2010; Thiagarajan et al. 2011) imparting the possibility that the method was in general little influenced by vital effects and other non-equilibrium phenomena that confound conventional oxygen isotope thermometry and Mg/Ca thermometry. This view however began to unravel as further studies demonstrated subtle non-equilibrium effects the clumped isotopic composition of some corals (Thiagarajan *et al.* 2011, Saenger *et al.* 2012) and molluscs (Henkes et al. 2013).

The main aim of this research was to assess how robust the clumped isotope thermometer is to biologically mediated non-equilibrium precipitation. This is achieved through sections 1-3 of the thesis, summarised in the following paragraphs. The $^{13}\text{C}^{18}\text{O}^{16}\text{O}$ isotopologue of interest in carbonate clumped isotope thermometry has an exceptionally rare abundance thus, in order to ensure precision of measurement, this study first focused is on (1) methodological development, primarily CO_2 purification and recognition of sample contamination. Following this, through the identification of non-equilibrium precipitation in Δ_{47} of previously under-investigated groups such as (2) the echinoids and (3) modern cephalopods we highlight the pervasive nature of non-equilibrium clumped isotopic signatures in biogenic carbonate. This study, initiated in 2014, in conjunction with other research that has been presented in the intervening time period (Kimball et al., Spooner et al., Banjai et al. 2018) indicates that vital effects in the clumped isotope composition of biogenic carbonate are much more widespread than initially thought and should be accounted for in Δ_{47} based paleoenvironmental reconstructions.

Finding 1: Impact of sample contamination on Δ_{47} values of analyte CO_2 and improved methods of CO_2 purification for clumped isotope analysis

The $^{13}\text{C}^{18}\text{O}^{16}\text{O}$ isotopologue in analyte CO_2 has a natural abundance of only 44.4 ppm at 25°C; thus, small isobaric effects on mass 47 can have a significant impact on the calculated

carbonate formation temperature. Carbonate clumped isotope thermometry has recently been applied to an increasing variety of samples including biological carbonates formed on an organic matrix (this study) meteorites (Guo and Eiler, 2007; Halevy et al., 2011) cataclasites (Swanson et al., 2012) and concretions (Loyd et al., 2012; Dale et al., 2014). These samples often contain high concentrations of organic compounds, sulfur and sulfur compounds, which may be volatilized on acid digestion to produce short-chain organic molecules and/or sulfide gases. This study illustrates the relationship between the metric Δ_{47} and contamination parameters $\Delta_{48\text{offset}}$ and the 49 parameter, contributing to the further development of reliable indicators of contamination. It is thus useful to the clumped isotope community as a whole especially those seeking to make clumped isotope measurements on “dirty” or contaminated samples such as those discussed above. The novel finding that reducing the static Porapak-Q (PPQ) trap temperature to -60°C can result in improved carbon dioxide purification for contaminant-rich samples may be useful for laboratories purifying CO_2 through a static PPQ trap without the use of a helium carrier gas. This includes those using automated sample devices such as the modified Thermo Scientific Kiel IV14 and small sample preparation inlets. Furthermore improvements in data quality assessment with regards to sample contamination allows us to be more confident in the diagnosis of small offsets in the Δ_{47} of biogenic carbonate in sections 2 and 3 as “true” offsets and not the result of sample contamination. Moreover provides a concrete methodology, which can be used as a springboard for further study.

Finding 2. “Vital effects” in the clumped (^{13}C - ^{18}O) isotope composition of echinoid calcite

Echinoderms, despite their complex inter-cellular bio-mineralization strategy, large inter-skeletal fractionation of $\delta^{18}\text{O}$, $\delta^{13}\text{C}$ and rapid calcite precipitation had however not been previously investigated with regards to their clumped isotope composition. These features make echinoderms and excellent target group to test for and investigate the nature of vital effects in Δ_{47} of $\delta^{18}\text{O}$, $\delta^{13}\text{C}$. In this study we specifically focus on the echinoids, both regular and irregular.

This study illustrates a positive offset in Δ_{47} of on average 0.014‰ relative to the inorganic calibration of the clumped isotope thermometer, corresponding to approximately a 4.5°C underestimation of precipitation temperature at 15°C . The observed offset is statistically robust at $0.0146 \pm 0.0042\text{‰}$ and is consistent across all growth temperatures and echinoid

species measured indicating it may be corrected by deducting 0.015‰ from echinoid Δ_{47} values prior to applying the inorganic Δ_{47} -temperature calibration. Echinoids have a unique strategy of intercellular biomineralization, depositing high magnesium calcite (Chave, 1954; Schroeder et al., 1969; McClintock et al., 2011) on an organic matrix with an initial phase of ACC deposition (Markel et al., 1986). Observed enrichments in Δ_{47} and $\delta^{18}\text{O}$ are tentatively linked to pH effects on DIC speciation in the calcifying fluid in association with rapid precipitation rate and/or ACC precipitation on an organic matrix. Intercellular biomineralization in echinoderm tissue would serve an important function in isolating the site of biomineral formation from the external environment facilitating the inheritance of disequilibrium Δ_{47} by the resultant mineral (Wiener and Dove). In this, this study makes its first provisional link between “vital effects” and biomineralization mechanism. The addition of a further group to the growing list of organisms showing statistically distinguishable “vital effects” in Δ_{47} is evidence that vital effects in the clumped isotope composition of biogenic carbonate are more widely spread than at first conceived.

Finding 3. Taxonomic non-equilibrium effects in clumped (^{13}C - ^{18}O) isotope composition of modern cephalopods

This section differs from the previous study in re-visiting a group that previously has been shown to exhibit non-equilibrium Δ_{47} signatures (Dennis et al. 2013) albeit at a time prior to recent advances in data processing and inter-laboratory comparison. The interest in the initial Dennis et al. (2013) study was that it advocated assumed equilibrium precipitation in $\delta^{18}\text{O}$ in conjunction with significant offsets in Δ_{47} from the inorganic calibration of Ghosh et al. (2006). This study sought to address discrepancies in cephalopod Δ_{47} values in greater detail and within the framework of modern clumped isotope methodology, including the ^{17}O correction parameters of Brand et al. and use of the previously developed combined inorganic equation of Davies and John (2018) as a baseline from which to determine deviations in Δ_{47} . This study illustrates clumped isotopic compositions corresponding to on average a 10°C overestimate in shell formation temperature in modern nautili, with Δ_{47} displaying correlation with $\delta^{18}\text{O}$. This finding is important as further elucidates the link between isotopic disequilibrium in $\delta^{18}\text{O}$ and Δ_{47} providing a further case study in which disequilibrium in one system is reflected in the other most likely as a result of a common mechanism. This study also illustrates inter skeletal variation in cephalopod Δ_{47} with a decrease in Δ_{47} from juvenile to adult septa the clumped isotope composition of most recently formed septa yield growth temperatures 3-8°C above the upper temperature limit of survival of the organism (27°C). Non-equilibrium clumped

isotope signatures may occur as a result of variations in salinity in cameral fluid within nautili chambers, dehydration of HCO_3^- and/or isotopic effects relating to CO_2 diffusion out of the extrapallial fluid. This research highlights the need for caution in the indiscriminate application of the clumped isotope thermometer to fossil cephalopods.

2) WIDER IMPLICATIONS

This thesis forms part of a growing body of literature illustrating the need for group specific Δ_{47} -temperature calibration of the clumped isotope thermometer. Furthermore investigation of the Δ_{47} composition of modern cephalopod molluscs has resulted in re-evaluation of the degree to which modern cephalopods, and by association fossil cephalopods, deposit their shells in oxygen isotope equilibrium with the surrounding environment.

Previous study have demonstrated the impact of kinetic effects during the hydration/hydroxylation of CO_2 on stable oxygen and clumped isotopic composition (McConnaughey, 1989a; McConnaughey, 1989b; Saenger et al., 2012; Bajnai et al., 2018). A further implication of this research is to highlight the potential for metabolic/taxonomic control on vital effects in Δ_{47} . We present data indicating that offsets in Δ_{47} from the inorganic calibration may be assiduously linked with pH and/or salinity effects. Attempts to identify, quantify and correct for vital effects in Δ_{47} should therefore include but not be limited groups likely impacted by kinetic effects i.e those with rapid growth rates at low temperatures.

Comparison between the echinoids and cephalopods indicate that offsets in Δ_{47} from the inorganic calibration (eg Davies and John 2018) may be easier to correct for in some groups (echinoids) than others (nautili), a factor that must be taken into account when selecting target species on which to apply the technique. This work does not diminish the utility of the clumped isotope thermometer in palaeoclimate reconstruction. It rather highlights the need to understand the mechanisms influencing the isotopic identity of biogenic carbonates prior to applying isotopic tools to the fossil record.

3) FURTHER WORK

The results and discussions in this thesis have drawn attention to the role of “vital effects” in the clumped isotopic composition of biogenic carbonates. The mechanisms that cause these effects however remain, to a certain degree, ambiguous. Future considerations could be

focused on further linking vital effects in echinoderms and molluscs with mechanistic processes enabling us to be more predictive in diagnosing in which further groups/species they will manifest and enabling correction for these effects. Further investigations following on from this research may thus be focused on (1) improvements in sample pre-treatment and CO₂ purification for clumped isotope analysis (2) understanding the impacts of calcifying fluid pH on Δ_{47} and $\delta^{18}\text{O}$ (3) determining the effects of organic additives in the calcifying fluid, ACC formation and subsequent matrix driven biomineralization on Δ_{47} and $\delta^{18}\text{O}$.

3.1 Improvements in sample pre-treatment and CO₂ purification of clumped isotope analysis

In chapter 3 we discuss improvements CO₂ purification using a static Porapak-Q (PPQ) trap and application of contamination indices Δ_{48} offset and 49 parameter. Further work could be focused on sample pre-treatment methods prior to acidification such as low temperature oxygen plasma ashing following (Lebeau et al., 2014) and in comparison with established pre-treatment methods such as H₂O₂ treatment (Eagle et al., 2010). Further work could also be directed at determining differences in the efficacy of CO₂ purification using a static PPQ trap in comparison with active flow through a GC column using He as a carrier gas as employed in the newly developed IBEX. This may include but not be limited to differences between the two methods in absolute Δ_{48} offset and 49 parameter of selected contaminated samples, memory effects of the PPQ trap/GC, and appropriate “cleaning” of the PPQ trap/GC between samples.

3.2 The effect of pH of the calcifying fluid on Δ_{47} and $\delta^{18}\text{O}$

In chapter 4 it is postulated that enrichment in echinoid Δ_{47} from expected values may relate to pH of the calcifying fluid (pH_{cf}) from which the mineral formed in conjunction with direct inheritance of DIC Δ_{63} composition. This may be further investigated by making measurements of pH_{cf} direct or indirect in echinoderms and other groups, particularly those which biomineralize from an isolated fluid. pH_{cf} may be made indirectly determined using the boron isotope proxy, $\delta^{11}\text{B}$ of carbonates may be linked with pH of the parent fluid

$$pH = pK_B - \log \left(\frac{\delta^{11}B_{sw} - \delta^{11}B_c}{\alpha_{4-3}^{-1} \times \delta^{11}B_c - \delta^{11}B_{sw} + 1000 \times (\alpha_{4-3}^{-1} - 1)} \right) \quad (\text{Equation 6.1})$$

where pK_B is the dissociation constant between B(OH)₃ and, $\delta^{11}\text{B}_c$ is the boron isotopic composition of the carbonates $\delta^{11}\text{B}_{sw}$ the boron isotopic composition of seawater and α_{4-3} the fractionation factor between B(OH)₃ and B(OH)₄⁻ (Pagani et al., 2005, Klochko et al., 2006,

Rollion-Bard and Erez, 2010). This has previously been demonstrated for pH_{cf} corals (McCulloch et al., 2012; Holcomb et al., 2014; Sutton et al., 2017). Complicating variable vital effects on $\delta^{11}\text{B}_c$ may however limit the utility of this approach. A more direct method may be to make microelectrode measurements (Al-Horani et al., 2002; Ries, 2011) or use pH sensitive dyes (Venn et al., 2011) to determine pH_{cf}. In this way the relationship between pH_{cf}, carbonate saturation state (Ω), Δ_{47} and $\delta^{18}\text{O}$ may be further determined.

3.3 The effect of organic additives, ACC formation and subsequent matrix driven biomineralization on Δ_{47} and $\delta^{18}\text{O}$

Echinoids and cephalopod molluscs are thought to undergo organic matrix controlled biomineralization (Ameys et al., 1998; Weiner and Addadi, 2011; Immenhauser et al., 2015). Matrix macromolecules have been demonstrated to be responsible for the formation and stabilization of ACC, conversion to calcite occurring through conversion or rearrangement of these molecules (Raz et al., 2003). In vitro carbonate precipitation experiments from solutions including additives such as L-aspartic acid sodium monohydrate, L-glutamic acid sodium monohydrate oxydiacetic acid, citric acid and malonic acid (Wang et al., 2009) proceeded by subsequent measurement of carbonate Δ_{47} composition may further elucidate if organic matrix macromolecules effect the Δ_{47} composition of carbonate. This approach however requires precipitation of enough material (approximately 15 mg carbonate) on which carry out the clumped isotope measurement.

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APPENDIX

Chapter 3 Isotopic Data

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Chapter 4 Isotopic Data

	ID	Mass Spec	d18O VPDB (Final)	d18O VPDB (Final) SE	d13C VPDB (Final)	d13C VPDB (Final) SE	Clumped AFF	D47 CDES (Final)	D47 CDES (Final) SE	D47 CDES (Final) SD	D48 Offset	49 Param
S1	BS		-1.39	0.06	0.01	0.06		0.706	0.005	0.009		
R1	2016-06-13 16:48 BST	Pinta	-1.29		0.08		0.082	0.707			0.121	0.081
R2	2016-06-14 15:43 BST	Pinta	-1.38		0.06		0.082	0.714			-0.075	0.065
R3	2016-06-16 16:25 BST	Pinta	-1.51		-0.1		0.082	0.696			-0.093	0.076
S2	BT		-1.75	0.1	-2.03	0.02		0.72	0.004	0.006		
R1	2016-06-14 17:53 BST	Pinta	-1.64		-2.04		0.082	0.722			-0.015	0.176
R2	2016-06-16 14:17 BST	Pinta	-1.94		-2.06		0.082	0.713			-0.128	0.051
R3	2016-06-16 22:54 BST	Pinta	-1.66		-1.98		0.082	0.725			0.489	0.074
S3	GA1		-1.59	0.04	1.15	0.02		0.702	0.005	0.009		
R1	2016-05-11 19:05 BST	Pinta	-1.63		1.12		0.082	0.697			0.349	0.162
R2	2016-06-13 11:49 BST	Pinta	-1.63		1.16		0.082	0.696			-0.188	0.106
R3	2017-06-07 19:06 BST	Pinta	-1.52		1.17		0.082	0.713			0.472	0.053
S4	GA2		-1.39	0.05	1.2	0.02		0.696	0.005	0.008		
R1	2016-06-13 14:45 BST	Pinta	-1.33		1.2		0.082	0.698			0.245	0.089
R2	2016-06-16 18:23 BST	Pinta	-1.48		1.17		0.082	0.687			-0.014	0.09

R3	2016-06-17 16:06 BST	Pinta	-1.34		1.24		0.082	0.703		0.209	0.216
S5	GLT		-1.69	0.07	0.67	0.02		0.705	0.011	0.024	
R1	2016-05-04 17:09 BST	Pinta	-1.78		0.64		0.082	0.686		-0.226	0.076
R2	2016-05-09 22:29 BST	Pinta	-1.75		0.61		0.082	0.692		-0.066	0.091
R3	2016-06-12 13:28 BST	Pinta	-1.84		0.67		0.082	0.716		-0.088	0.098
R4	2016-07-21 17:57 BST	Pinta	-1.66		0.75		0.082	0.742		0.19	0.126
R5	2016-08-07 22:47 BST	Pinta	-1.43		0.68		0.082	0.688		0.339	0.022
S6	GS		-1.64	0.18	0.28	0.01		0.688	0.011	0.019	
R1	2016-04-27 15:58 BST	Pinta	-1.56		0.3		0.082	0.68		-0.601	0.039
R2	2016-05-04 19:23 BST	Pinta	-1.98		0.25		0.082	0.674		-0.352	-0.012
R3	2016-08-07 20:40 BST	Pinta	-1.37		0.29		0.082	0.709		0.251	0.023
S7	GT		-1.31	0.17	1.24	0.05		0.702	0.012	0.022	
R1	2016-05-04 15:09 BST	Pinta	-1.57		1.2		0.082	0.701		-0.11	0.06
R2	2016-05-09 20:31 BST	Pinta	-1.38		1.18		0.082	0.724		-0.069	0.084
R3	2017-06-19 13:09 BST	Pinta	-0.99		1.35		0.082	0.681		-0.097	-0.062
S8	MA1		1.41	0.09	-0.19	0.05		0.764	0.006	0.01	
R1	2016-05-09 15:48 BST	Pinta	1.24		-0.3		0.082	0.775		0.151	0.109
R2	2016-05-11 16:56 BST	Pinta	1.44		-0.13		0.082	0.759		0.045	0.13
R3	2016-06-12 17:36 BST	Pinta	1.54		-0.14		0.082	0.757		-0.165	0.115
S9	MA2		1.49	0.1	-0.03	0.02		0.75	0.004	0.007	
R1	2016-11-21 12:16 GMT	Pinta	1.62		-0.03		0.082	0.748		-0.221	0.116

R2	2016-12-05 10:45 GMT	Pinta	1.55		-0.07		0.082	0.758		-0.106	0.055
R3	2016-12-05 21:14 GMT	Pinta	1.3		0.01		0.082	0.745		0.16	0.099
S10	MB		1.29	0.14	-0.16	0.01		0.736	0.005	0.009	
R1	2016-05-09 13:26 BST	Pinta	1.14		-0.18		0.082	0.726		-0.069	0.107
R2	2016-05-11 14:48 BST	Pinta	1.57		-0.14		0.082	0.739		-0.066	0.127
R3	2016-06-12 20:01 BST	Pinta	1.14		-0.15		0.082	0.743		-0.038	0.081
S11	ML		1.12	0.15	-1.35	0.01		0.746	0.01	0.016	
R1	2016-11-21 18:19 GMT	Pinta	1.13		-1.36		0.082	0.73		0.321	0.074
R2	2016-11-23 00:20 GMT	Pinta	0.85		-1.34		0.082	0.763		0.614	0.119
R3	2016-11-29 18:36 GMT	Pinta	1.38		-1.35		0.082	0.746		0.22	0.142
S12	MP		1.18	0.03	-0.69	0.05		0.756	0.012	0.02	
R1	2016-05-09 09:30 BST	Pinta	1.14		-0.66		0.082	0.779		0.113	0.142
R2	2016-05-11 12:47 BST	Pinta	1.16		-0.78		0.082	0.742		0.025	0.117
R3	2016-06-13 09:43 BST	Pinta	1.24		-0.62		0.082	0.747		-0.219	0.084
S13	MS		1.03	0.08	-0.55	0.01		0.736	0.006	0.01	
R1	2016-11-17 20:10 GMT	Pinta	0.97		-0.53		0.082	0.726		-0.029	0.045
R2	2016-11-21 14:14 GMT	Pinta	0.94		-0.56		0.082	0.747		0.218	0.105
R3	2016-11-22 21:44 GMT	Pinta	1.19		-0.56		0.082	0.734		0.315	0.102
S14	SDA1		-0.31	0.06	-3.22	0.03		0.765	0.005	0.009	
R1	2017-02-11 22:27 GMT	Pinta	-0.34		-3.28		0.082	0.767		1.148	0.049
R2	2017-02-19 15:07 GMT	Pinta	-0.19		-3.21		0.082	0.755		0.052	-0.042

R3	2017-02-21 12:47 GMT	Pinta	-0.38		-3.18	0.082	0.773		-0.744	-0.102
S15	SDA2		-0.23	0.03	-3.02	0.05	0.768	0.008	0.013	
R1	2017-02-19 17:26 GMT	Pinta	-0.19		-2.95	0.082	0.756		0.072	-0.046
R2	2017-02-28 12:38 GMT	Pinta	-0.28		-3.01	0.082	0.766		0.992	0.157
R3	2017-03-08 14:52 GMT	Pinta	-0.23		-3.1	0.082	0.782		0.234	0.128
S16	SDLT		0.03	0.08	-2.4	0.02	0.741	0.005	0.008	
R1	2017-02-11 17:37 GMT	Pinta	0		-2.41	0.082	0.746		0.215	0.017
R2	2017-02-12 16:25 GMT	Pinta	-0.1		-2.43	0.082	0.745		1.889	0.072
R3	2017-02-18 16:57 GMT	Pinta	0.19		-2.38	0.082	0.731		0.075	-0.167
S17	SDS		0.11	0.1	-1.34	0.01	0.753	0.012	0.02	
R1	2017-02-11 14:59 GMT	Pinta	-0.08		-1.35	0.082	0.773		0.216	0.022
R2	2017-02-12 11:18 GMT	Pinta	0.24		-1.32	0.082	0.754		0.534	0.093
R3	2017-06-19 15:23 BST	Pinta	0.19		-1.34	0.082	0.732		-0.093	-0.011
S18	SDT		-0.5	0.11	-3.47	0.03	0.758	0.01	0.019	
R1	2017-02-11 20:09 GMT	Pinta	-0.56		-3.49	0.082	0.751		0.359	0.052
R2	2017-02-12 18:48 GMT	Pinta	-0.26		-3.38	0.082	0.778		1.224	0.081
R3	2017-02-18 12:04 GMT	Pinta	-0.77		-3.52	0.082	0.767		-0.212	-0.109
R4	2017-02-19 12:53 GMT	Pinta	-0.4		-3.5	0.082	0.734		0	-0.031
S19	YA1		0.86	0.13	-1.22	0.01	0.701	0.012	0.021	
R1	2016-11-07 21:32 GMT	Pinta	1.03		-1.24	0.082	0.687		0.308	0.113
R2	2016-11-14 14:30 GMT	Pinta	0.93		-1.23	0.082	0.691		-0.185	0.048

R3	2016-11-17 16:03 GMT	Pinta	0.6		-1.2	0.082	0.726		0.225	0.144
S20	YA2		0.7	0.07	-1.5	0.04	0.687	0.015	0.03	
R1	2016-11-07 13:29 GMT	Pinta	0.67		-1.54	0.082	0.714		0.117	0.158
R2	2016-11-09 12:23 GMT	Pinta	0.5		-1.58	0.082	0.651		-1.372	0.116
R3	2016-11-14 16:42 GMT	Pinta	0.83		-1.42	0.082	0.676		0.259	0.005
R4	2017-06-19 17:35 BST	Pinta	0.78		-1.44	0.082	0.709		1.159	0.026
S21	YLP		1.33	0.05	-1.51	0.08	0.722	0.015	0.025	
R1	2016-11-07 15:32 GMT	Pinta	1.42		-1.66	0.082	0.698		0.029	0.113
R2	2016-11-21 16:10 GMT	Pinta	1.33		-1.44	0.082	0.718		0.347	0.123
R3	2016-11-29 14:08 GMT	Pinta	1.25		-1.42	0.082	0.749		0.047	0.185
S22	YLR		1	0.05	-2.22	0.01	0.716	0.006	0.01	
R1	2016-11-09 20:50 GMT	Pinta	1.11		-2.23	0.082	0.728		0.358	0.191
R2	2016-11-14 12:24 GMT	Pinta	0.95		-2.19	0.082	0.708		-0.582	-0.002
R3	2016-11-17 18:06 GMT	Pinta	0.95		-2.23	0.082	0.712		0.026	0.123
S23	YLT		0.85	0.12	-0.93	0.03	0.711	0.011	0.018	
R1	2016-11-07 19:34 GMT	Pinta	1.08		-0.94	0.082	0.692		0.428	0.082
R2	2016-11-09 14:34 GMT	Pinta	0.72		-0.98	0.082	0.713		0.039	0.103
R3	2016-11-17 13:53 GMT	Pinta	0.74		-0.87	0.082	0.729		0.071	0.06
S24	YS		1.79	0.21	0.78	0.02	0.717	0.01	0.017	
R1	2016-10-04 11:55 BST	Pinta	1.38		0.81	0.082	0.717		-0.18	0.081
R2	2016-10-05 15:13 BST	Pinta	1.9		0.8	0.082	0.699		-0.619	-0.152

R3	2016-11-07 17:30 GMT	Pinta	2.08		0.74		0.082	0.734		0.349	0.156
S25	YT		0.6	0.11	-1.67	0.01		0.725	0.005	0.009	
R1	2016-11-21 10:12 GMT	Pinta	0.63		-1.68		0.082	0.719		-0.216	0.016
R2	2016-11-29 16:13 GMT	Pinta	0.4		-1.66		0.082	0.735		0.031	0.09
R3	2017-06-20 16:57 BST	Pinta	0.78		-1.66		0.082	0.722		0.383	-0.058

Chapter 4

ID	Mass Spec	d18O VPDB (Final)	d18O VPDB (Final) SE	d13C VPDB (Final)	d13C VPDB (Final) SE	D47 CDES (Final)	D47 CDES (Final) SE	D47 CDES (Final) SD	D48 Offset	49 Param
ANNCO		0.85	0.02	0.75	0.08	0.628	0.013	0.023		
2018-03-10 17:20 GMT	Santa Maria	0.82		0.65		0.626			-0.146	0.014
2018-03-13 19:03 GMT	Santa Maria	0.87		0.91		0.607			0.31	0.172
2018-05-09 21:02 BST	Santa Maria	0.87		0.69		0.653			0.971	0.181
ANNCS1		0.94	0.21	1.32	0.17	0.673	0.004	0.008		
2018-02-24 11:54 GMT	Santa Maria	0.37		1.59		0.682			0.245	0.204
2018-03-25 17:10 BST	Santa Maria	1.22		1.06		0.663			0.284	0.159
2018-05-09 16:02 BST	Santa Maria	0.89		0.99		0.678			0.667	0.178
2018-06-03 02:25 BST	Pinta	1.29		1.66		0.671			-0.185	0.314
ANNCS10		0.94	0.23	0.76	0.26	0.688	0.017	0.034		
2018-02-25 12:33 GMT	Santa Maria	0.73		0.38		0.66			-0.025	0.18
2018-03-06 17:00 GMT	Santa Maria	0.44		0.48		0.68			0.34	0.148

2018-05-09 18:32 BST	Santa Maria	1.03		0.67		0.676			1.568	0.236
2018-06-05 19:28 BST	Pinta	1.54		1.51		0.737			-0.091	0.248
ANNCS12		1.47	0.07	0.81	0.02	0.719	0.003	0.005		
2018-05-22 10:28 BST	Santa Maria	1.39		0.77		0.713			0.121	0.112
2018-05-23 09:37 BST	Pinta	1.6		0.84		0.722			-0.533	0.42
2018-05-23 10:30 BST	Santa Maria	1.41		0.82		0.723			0.034	0.046
ANNCS15		1.45	0.13	0.66	0.03	0.708	0.005	0.01		
2018-02-24 20:46 GMT	Santa Maria	1.22		0.59		0.7			-0.302	0.18
2018-03-06 21:35 GMT	Santa Maria	1.69		0.71		0.705			0.351	0.175
2018-03-25 21:39 BST	Santa Maria	1.44		0.68		0.718			0.273	0.22
ANNCS17		1.34	0.11	1.53	0.22	0.731	0.007	0.012		
2018-05-22 13:01 BST	Santa Maria	1.23		1.32		0.737			0.354	0.179
2018-05-23 12:36 BST	Santa Maria	1.23		1.3		0.738			0.552	0.102
2018-06-06 07:29 BST	Pinta	1.55		1.96		0.717			-0.023	0.222
ANNCS3		0.94	0.17	1.7	0.04	0.663	0.008	0.019		
2018-05-18 19:06 BST	Santa Maria	0.4		1.72		0.669			1.924	0.239
2018-05-23 05:06 BST	Pinta	1.15		1.76		0.65			-0.657	0.422
2018-05-23 17:30 BST	Santa Maria	0.7		1.56		0.663			0.646	0.08
2018-05-29 20:58 BST	Pinta	1.16		1.74		0.642			-0.368	0.306
2018-06-03 11:06	Pinta	1.26		1.71		0.691			-0.188	0.369

BST										
ANNCS5		1.2	0.15	1.33	0.03	0.693	0.009	0.02		
2018-03-06 14:22 GMT	Santa Maria	0.62		1.3		0.658			0.623	0.155
2018-03-08 11:29 GMT	Santa Maria	1.36		1.33		0.706			0.63	0.178
2018-03-25 19:27 BST	Santa Maria	1.23		1.22		0.699			0.018	0.18
2018-05-30 04:02 BST	Pinta	1.36		1.39		0.696			-0.326	0.302
2018-06-03 06:47 BST	Pinta	1.4		1.41		0.706			-0.102	0.312
ANNCS7		1.3	0.14	1.87	0.04	0.693	0.018	0.031		
2018-05-23 07:22 BST	Pinta	1.41		1.92		0.691			-0.44	0.403
2018-05-23 19:56 BST	Santa Maria	1.03		1.78		0.663			0.398	0.075
2018-05-30 01:40 BST	Pinta	1.46		1.91		0.725			-0.358	0.344
ANPUO		0.92	0.15	0.34	0.06	0.654	0.018	0.036		
2018-05-22 18:18 BST	Santa Maria	1		0.3		0.636			1.419	0.136
2018-05-23 23:24 BST	Pinta	1.05		0.18		0.612			-0.233	0.405
2018-05-29 16:22 BST	Pinta	1.17		0.44		0.69			-0.433	0.313
2018-06-02 15:18 BST	Santa Maria	0.48		0.44		0.676			0.735	0.186
ANPUS1		0.91	0.16	1.03	0.05	0.684	0.008	0.013		
2018-03-14 16:56 GMT	Santa Maria	1.21		1.04		0.698			0.738	0.178
2018-03-23 17:51 GMT	Santa Maria	0.69		1.11		0.672			0.067	0.021
2018-03-24 13:33 GMT	Santa Maria	0.84		0.95		0.682			0.359	0.078

ANPUS10		1.42	0.04	1.21	0.16	0.698	0.005	0.01		
2018-03-23 15:09 GMT	Santa Maria	1.36		1.5		0.709			0.017	0.044
2018-03-27 16:25 BST	Santa Maria	1.33		1.41		0.698			0.47	0.178
2018-04-29 19:44 BST	Santa Maria	1.58		1.45		0.684			0.281	0.139
2018-05-23 18:44 BST	Pinta	1.4		0.75		0.693			-0.381	0.402
2018-05-29 18:45 BST	Pinta	1.42		0.91		0.706			-0.34	0.316
ANPUS15		1.3	0.12	1.16	0.21	0.717	0.008	0.013		
2018-03-14 20:57 GMT	Santa Maria	1.09		1.55		0.708			0.705	0.171
2018-03-26 19:37 BST	Santa Maria	1.51		0.83		0.732			0.348	0.192
2018-05-11 13:25 BST	Santa Maria	1.31		1.11		0.712			0.75	0.193
ANPUS5		1.11	0.18	0.84	0.03	0.676	0.004	0.007		
2018-03-23 12:34 GMT	Santa Maria	0.79		0.79		0.683			0.368	0.037
2018-04-29 13:35 BST	Santa Maria	1.15		0.88		0.675			0.478	0.11
2018-05-11 11:09 BST	Santa Maria	1.4		0.84		0.67			0.639	0.207
ANWA		-0.74	0.4	-2.28	0.17	0.695	0.07	0.099		
2018-05-24 11:54 BST	Santa Maria	-1.14		-2.45		0.764			0.517	-0.037
2018-07-26 22:30 BST	Pinta	-0.34		-2.11		0.625			-0.428	0.239
NFI		0.98	0.14	1.94	0.22	0.702	0.002	0.003		
2017-06-20 00:51 BST	Pinta	0.7		2		0.7			0.087	0.007
2017-06-28 17:20 BST	Pinta	1.15		2.28		0.7			0.18	0.072

2018-03-06 23:58 GMT	Santa Maria	1.09		1.54		0.705			0.292	0.226
NLI		0.8	0.24	1.59	0.03	0.698	0.013	0.022		
2017-06-16 14:14 BST	Pinta	0.32		1.54		0.707			0.448	0.147
2017-06-19 22:21 BST	Pinta	1.05		1.64		0.673			0.071	0.034
2017-06-28 12:33 BST	Pinta	1.03		1.58		0.713			0.057	0.084
NNCL		0.32	0.05	2.08	0.04	0.695	0.013	0.022		
2018-05-22 15:53 BST	Santa Maria	0.31		2.08		0.71			1.111	0.332
2018-05-23 14:59 BST	Santa Maria	0.24		2.01		0.705			0.695	0.099
2018-05-23 16:30 BST	Pinta	0.43		2.15		0.67			-0.331	0.391
NNCS		0.88	0.05	1.48	0.02	0.682	0.007	0.012		
2017-06-15 21:26 BST	Pinta	0.96		1.52		0.695			0.59	0.196
2017-06-19 19:46 BST	Pinta	0.78		1.48		0.678			0.023	0.03
2017-06-28 14:47 BST	Pinta	0.89		1.45		0.673			0.147	0.068
NTB1		0.47	0.05	1.74	0.3	0.704	0.009	0.016		
2017-06-13 12:27 BST	Pinta	0.37		1.14		0.702			0.256	0.259
2017-06-15 12:15 BST	Pinta	0.54		1.95		0.721			0.048	0.123
2017-06-16 11:18 BST	Pinta	0.51		2.12		0.689			0.076	0.043
NTB2		0.75	0.09	1.22	0.04	0.671	0.008	0.014		
2017-06-13 19:13 BST	Pinta	0.6		1.18		0.657			0.052	0.065
2017-06-14 20:21 BST	Pinta	0.9		1.3		0.669			0.077	0.149

2017-06-15 14:40 BST SO1	Pinta	0.73		1.18		0.686			0.891	0.219
2018-03-08 20:10 GMT	Santa Maria	1.16	0.18	-2.89	0.06	0.744	0.009	0.019		
2018-03-10 12:43 GMT	Santa Maria	1.36		-2.9		0.735			0.532	0.147
2018-03-11 15:10 GMT	Santa Maria	0.74		-2.9		0.729			-0.074	-0.003
2018-03-13 14:13 GMT	Santa Maria	1.52		-2.74		0.77			0.061	0.152
2018-03-14 14:38 GMT	Santa Maria	1.04		-3.01		0.744			0.51	0.194
2018-05-22 22:17 BST	Santa Maria	1.07	0.05	-2.89	0.11	0.751	0.008	0.013		
2018-05-24 01:44 BST	Santa Maria	1.12		-2.7		0.766			0.593	0.203
2018-05-24 03:58 BST	Pinta	0.97		-3.07		0.741			-0.488	0.44
2018-05-24 17:24 GMT	Pinta	1.12		-2.9		0.745			-0.097	0.406
2018-05-22 19:55 BST	Pinta	0.03	0.05	-3.51	0.16	0.705	0.006	0.01		
2017-04-07 18:41 BST	Santa Maria	0.12		-3.19		0.716			0.4	0.155
2017-06-06 12:47 BST	Pinta	-0.01		-3.65		0.702			-0.818	0.452
2017-06-07 12:18 BST	Pinta	-0.03		-3.68		0.697			-0.033	0.417
2017-05-02 15:20 BST	Pinta	0.33	0.11	1.79	0.03	0.676	0.006	0.011		
2017-06-06 12:47 BST	Pinta	0.2		1.76		0.671			0	-0.023
2017-06-07 12:18 BST	Pinta	0.55		1.86		0.669			0.036	0.129
2017-06-07 12:18 BST	Pinta	0.24		1.77		0.688			0.093	0.194
2017-05-02 15:20 BST	Pinta	0.54	0.17	0.72	0.13	0.696	0.003	0.006		
2017-05-02 15:20 BST	Pinta	0.88		0.97		0.689			0.237	0.082

2017-06-13 16:56 BST	Pinta	0.43		0.57		0.7			0.041	0.101
2017-06-14 13:22 BST	Pinta	0.33		0.63		0.698			-0.034	0.131
WNPUO3		0.58	0.06	0.58	0.09	0.668	0.009	0.015		
2017-06-13 21:58 BST	Pinta	0.68		0.53		0.651			0.375	0.09
2017-06-14 15:34 BST	Pinta	0.48		0.46		0.68			0.064	0.091
2017-06-15 19:08 BST	Pinta	0.57		0.74		0.672			0.172	0.09
WNPUO4		0.17	0.08	-1	0.02	0.672	0.023	0.04		
2017-06-06 17:55 BST	Pinta	0.24		-0.98		0.649			0.155	0.135
2017-06-07 14:30 BST	Pinta	0.02		-1		0.65			-0.065	0.079
2017-06-13 14:45 BST	Pinta	0.25		-1.04		0.718			0.318	0.23
WNPUO5		0.44	0.11	1.69	0.04	0.677	0.019	0.033		
2017-06-07 16:48 BST	Pinta	0.22		1.72		0.653			-0.041	-0.013
2018-03-08 13:34 GMT	Santa Maria	0.53		1.61		0.714			0.523	0.186
2018-03-10 14:53 GMT	Santa Maria	0.57		1.73		0.664			-0.044	0.068
WNPUS1		0.66	0.1	1.3	0.06	0.673	0.004	0.007		
2018-05-31 00:18 BST	Pinta	0.78		1.27		0.679			-0.238	0.333
2018-05-31 14:01 BST	Santa Maria	0.45		1.22		0.666			1.135	0.152
2018-06-03 16:57 BST	Pinta	0.75		1.42		0.673			-0.245	0.327
WNPUS13		1.09	0.14	-0.31	0.21	0.72	0.024	0.048		
2018-05-24 16:57 BST	Santa Maria	0.66		-0.24		0.784			1.275	0.02

2018-06-02 12:02 BST	Pinta	1.17		-0.04		0.719			-0.503	0.307
2018-06-03 00:23 BST	Pinta	1.23		-0.04		0.707			-0.195	0.29
2018-06-06 14:29 BST	Pinta	1.3		-0.91		0.669			-0.153	0.214
WNPUS17		1.24	0.09	-0.71	0.18	0.736	0.015	0.034		
2018-05-25 00:12 BST	Santa Maria	1.39		-1		0.794			0.332	0.059
2018-05-30 06:22 BST	Pinta	0.97		-0.62		0.721			-0.374	0.333
2018-05-30 15:03 BST	Pinta	1.08		-0.05		0.716			-0.274	0.327
2018-06-02 15:15 BST	Pinta	1.3		-0.93		0.713			-0.356	0.275
2018-06-06 16:55 BST	Pinta	1.47		-0.96		0.734			-0.041	0.262
WNPUS21		1.26	0.07	-0.89	0.04	0.697	0.009	0.016		
2018-05-30 17:17 BST	Pinta	1.13		-0.97		0.691			-0.398	0.309
2018-05-31 20:56 BST	Santa Maria	1.32		-0.87		0.686			0.379	0.177
2018-06-02 17:29 BST	Pinta	1.33		-0.83		0.715			-0.29	0.28
WNPUS25		0.74	0.31	-0.37	0.25	0.708	0.025	0.049		
2018-05-30 19:45 BST	Pinta	-0.12		0.15		0.689			-0.399	0.281
2018-05-31 23:14 BST	Santa Maria	0.87		-0.82		0.647			0.044	0.079
2018-06-02 22:00 BST	Pinta	0.85		-0.78		0.752			-0.253	0.297
2018-06-06 09:43 BST	Pinta	1.35		-0.04		0.745			-0.044	0.231
WNPUS5		1.19	0.12	-0.97	0.09	0.702	0.012	0.021		
2018-05-31 02:31	Pinta	0.99		-0.81		0.711			-0.181	0.349

BST									
2018-05-31 16:13	Santa Maria	1.19		-1.12		0.718		0.801	0.154
BST									
2018-06-02 19:45	Santa Maria	1.4		-0.97		0.678		0.22	0.191
BST									
WNPUS9		0.87	0.08	0.42	0.13	0.724	0.021	0.041	
2018-05-24 21:36	Santa Maria	0.75		0.06		0.698		-0.353	-0.121
BST									
2018-05-31 04:53	Pinta	0.91		0.65		0.702		-0.107	0.356
BST									
2018-05-31 18:37	Santa Maria	0.75		0.43		0.786		1.47	0.179
BST									
2018-06-03 19:15	Pinta	1.07		0.53		0.711		-0.244	0.27
BST									