An explanation for the ^{18}O excess in Noelaerhabdaceae coccolith calcite

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Abstract

Coccoliths have dominated the sedimentary archive in the pelagic environment since the Jurassic. The biominerals produced by the coccolithophores are ideally placed to infer sea surface temperatures from their oxygen isotopic composition, as calcification in this photosynthetic algal group only occurs in the sunlit surface waters. In the present study, we dissect the isotopic mechanisms contributing to the "vital effect", which overprints the oceanic temperatures recorded in coccolith calcite. Applying the passive diffusion model of carbon acquisition by the marine phytoplankton widely used in biogeochemical and palaeoceanographic studies, our results suggest that the oxygen isotope offsets from inorganic calcite in fast dividing species *Emiliania huxleyi* and *Gephyrocapsa oceanica* originates from the legacy of assimilated ^{18}O-rich CO_2 that induces transient isotopic disequilibrium to the internal dissolved inorganic carbon (DIC) pool. The extent to which this intracellular isotopic disequilibrium is recorded in coccolith calcite (1.5 to +3 ‰ over a 10 to 25 °C temperature range) is set by the degree of isotopic re-equilibration between CO_2 and water molecules before intracellular mineralisation. We show that the extent of re-equilibration is, in turn, set by temperature through both physiological (dynamics of the utilisation of the DIC pool) and thermodynamic (completeness of the re-equilibration of the relative ^{18}O-rich CO_2 influx) processes. At the highest temperature, less ambient aqueous CO_2 is present for algal growth, and the consequence of carbon limitation is exacerbation of the oxygen isotope vital effect, obliterating the temperature signal. This culture dataset further demonstrates that the vital effect is variable for a given species / morphotype, and depends on the intricate relationship between the environment and the physiology of biomineralising algae.

Keywords:

Biomineralisation; *Emiliania huxleyi*; *Gephyrocapsa oceanica*; Oxygen isotope fractionation; Temperature proxy; Vital effect; Carbon (CO_2) limitation.

1. Introduction

Understanding how the physiology of marine organisms affects the fractionation of stable isotopes in biogenic carbonates has geological and biogeochemical importance. Firstly, such a goal would help the development and validation of geological proxies. Secondly, the stable isotope signatures determined from studies of fractionations then may be used to constrain present (and past) physiology, particularly with regard to carbon utilisation. In the case of the coccolithophores with the dual ability to fix carbon to produce organic matter and to calcify, the cells accumulate an internal carbon pool (*Ci*) to serve the carbon requirements both for photosynthetic carbon fixation in the chloroplast and mineralisation in the coccolith vesicle with large isotopic consequences (Sekino and Shiwaira, 1996; Rokitta and Rost, 2012; Benthien et al., 2007; Rickaby et al., 2010; Bolton and Stoll, 2013; Hermoso, 2014; Hermoso et al., 2014; Hermoso, 2015; Holtz et al., 2015). Previous culture studies have suggested a control of stable isotope composition (hence a vital effect) in coccolith calcite by algal growth rate (Ziveri et al., 2003). The vital effect is here defined as the species-specific offset in $\delta^{18}O$ values between biogenic calcite and inorganic calcite grown under the same conditions (temperature, pH, etc…). The aim of the present study is to deepen our knowledge of the mechanism of oxygen isotope fractionation in coccolith calcite by isolating the vital effect component and determining how its magnitude varies with growth rate, cell size, carbon availability and temperature. Carbon isotope data from cultured coccoliths are not presented in the present study, as they will be published in a subsequent contribution.

The early works by Anderson and Steinmetz (1981) and Paull and Thierstein (1987) documented a large modulation of the isotopic offset in Noelaerhabdaceae coccolith from the foraminiferal record through glacial / interglacial cycles in the Pleistocene. A recent reappraisal of the data identified a pCO_2-driver for such a modulation of the oxygen and carbon vital effects in coccoliths with maximum values during glacial (relatively low pCO_2) stades (Hermoso, 2016). Other sedimentary records suggest that the vital effect for reticulofenestrid coccoliths (extinct taxa of the Noelaerhabdaceae) was insignificant in the Pliocene (Bolton et al., 2012). During this period, pCO_2 values were similar to or higher than Pleistocene interglacial concentrations (Badger et al., 2013), hence representing a coherent CO_2 availability / palaeo-vital effect framework. Taken together, these results from the natural environment, however, challenge the constancy of the vital effect deduced from laboratory

culture experiments on Noelaerhabdaceae coccoliths (Dudley et al., 1986; Hermoso et al., 2015).

Here, we investigate how this vital effect varies using laboratory experiments. Our working hypothesis is that the vital effect derives from the isotopic disequilibrium of the *Ci* pool with regulating mechanisms such as cell geometry, temperature and CO_2 supply-to-demand ratio, as is the case for carbon isotopic fractionation in organic matter (Bidigare et al., 1997). We grew six strains of Noelaerhabdaceae coccolithophores with very distinct cell sizes and over a range of temperatures (Figs. 1 and 2). Changing temperature has a direct impact on the solubility of aqueous CO_2, on the isotopic equilibrium between the DIC species, DIC and water (Zeebe and Wolf-Gladrow, 2001). In addition, the physiology of algae also changes with temperature, in particular with regards to the carbon demand by the cells (Sett et al., 2014; Rickaby et al., 2016). Hence, by changing the ambient temperature of the culture medium, we test the consistency of temperature dependence of the magnitude of disequilibrium in the oxygen isotope system compared to equilibrium conditions, elucidate a component of the vital effect, and examine the reliability of the coccolith-derived $\delta^{18}O$ proxy for reconstructing sea surface temperatures (SSTs) in the past.

2. Material and methods

2.1. Medium

We used growth medium consisting of natural seawater from the English Channel sampled by the Marine Biology Association, Plymouth (UK). This seawater was aged at 4 °C for at least 6 months. Air was bubbled through each of the aged seawater batches at distinct temperatures (10; 15; 20; and 25 °C) overnight and prior to medium preparation. The pCO_2 of the laboratory air used to aerate the culture media was measured at 400 ppmV using a Licor LI-840A infrared analyser at 400 ppmV. Each batch was subsequently amended with nitrate (288 µM), phosphate (18 µM), EDTA, trace metals and vitamins following the *K*/2 recipe by Keller et al. (1987) and the pH adjusted to 8.2 (total scale) by addition of 1N NaOH. Prior to inoculation, medium was sterilised by microfiltration (Millipore 0.22 µm Stericup device) and transferred into sterile 0.25 L polystyrene culture flasks.

The initial concentration of aqueous CO_2 in each medium was calculated for each temperature using the software CO_2CALC (Robbins et al., 2010) with temperature, salinity, alkalinity and pH as input parameters. A total alkalinity of 2600 ± 39 µmol kg_{sw}^{-1} was measured in the initial batch solution using a 916 Ti Touch automatic titrator (Metrohm) and a 0.25 M HCl titrant standardised using a Na_2CO_3 solution. The method was adapted from Bradshaw et al (1981) for the determination of total alkalinity in seawater. Calculated concentrations of aqueous CO_2 in the medium ranged linearly from 13.5 µM at 10 °C to 8.3 µmol C kg_{sw}^{-1} at 25 °C (Fig. 2).

2.2. Cells and growth

Coccolithophore strains *Emiliania huxleyi* (RCC 1256; RCC 1216; RCC 1212) and *Gephyrocapsa oceanica* (RCC 1211; RCC 1318; RCC 1314) were supplied by the Roscoff Culture Collection, France. The assignment to a morphotype for *E. huxleyi* and typical coccosphere size are given in Fig. 1. These algal clones were selected to cover a large range of cell size within the Noelaerhabdaceae coccolithophores, ranging from 3.7 to 7.2 µm in diameter (Fig. 1). More information, including the origin and previous names of each strain can be found at: http://roscoff-culture-collection.org.

The experiments were conducted at the Oxford OceanBUG laboratory in March - June 2014. Algal growth took place in culture cabinets (Panasonic MLR-352) with an illuminated period of 14 h and a dark period of 10 h every day. In the culture cabinet, the light level at the position of the flasks was measured as 150 (±10) µmol photons.m^{-2}.s^{-1} using a QSL2101 light probe (Biospherical Instruments Inc.). Temperature loggers placed next to the culture flask in the incubator indicated that the precision of the target temperature was within a range of 0.2 °C.

Cells were acclimatised to the target temperature for at least ten generations prior to the start of the experiments with an initial concentration of ~ 200 cells mL^{-1}. To avoid culture artifacts arising from drift in the carbonate system chemistry ($[CO_{2\ aq}]$, pH) during the experiments (Langer et al., 2006; Barry et al., 2010; Hermoso, 2014), we cultured coccolithophores at very dilute cell densities, typically with utilisation of DIC less than 5 % during the course of the experiment. We also ensured that the final pH drifted less than 0.02 pH units. Each culture was implemented in duplicate.

Growth rates were calculated from measurement of cell density in the culture media at days 3 and 4 after inoculation. Cells were harvested at densities of ~ 6000 cells / mL for *Emiliania huxleyi* and ~ 10000 cells / mL of medium for *Gephyrocapsa oceanica*. The formula used to calculate μ from the evolution of cell density □□□□□□□□□□□□□□□□□□□□□□□□

$$\mu = \frac{\ln\,(cell\ conc\ final) - \ln\,(cell\ conc\ initial)}{\text{number of days between inital and final}} \qquad \text{(Eq. 1)}$$

where μ values are in day^{-1} and the cell concentrations are in number of cells per mL of medium.

Cell volumes were measured using a Beckman Coulter Counter Z2 analyser fitted with a 50 μm aperture tube after decalcification of the coccospheres with acid. The Z2 apparatus was calibrated daily using size-calibrated latex beads (diameter of the commercial batch provided by Beckman: 5.12 μm). Coccosphere diameters and counts were measured without any treatment with the same dilution factor (1:20). All these measurements were undertaken between 3 and 4 hours of the onset of the illuminated period set in the culture cabinets.

2.3. Isotopic analyses

Oxygen isotope ratios of calcite (coccolith) were measured at the *Institut des Sciences de la Terre de Paris* using a Kiel IV device attached to a Delta V Advantage mass spectrometer. Samples were first rinsed with neutralised deionised water to remove any trace of salt. Organic matter was removed by 10 % H_2O_2 (neutralised to pH 8 using 5M NaOH) left overnight at room temperature. The coccolith pellet was then washed three times by re-suspending in deionised water (pH adjusted to 7). Subsequently, an aliquot of ~ 100 μg of dried culture residues was reacted with 100 % anhydrous phosphoric acid at 70 °C in an individual vessel, and purified in an automated cryogenic distillation system to purify the CO_2 gas. Calibration of the results against the international standard NBS-19 was made via an in-house laboratory carbonate standard (a Carrara Marble). Reproducibility of replicated standards was usually better than 0.1 ‰ for $\delta^{18}O$ expressed in the VPDB scale.

In the present study, $\delta^{18}O_{calcite} - \delta^{18}O_{sw}$ (with $\delta^{18}O_{sw}$ constant and equal to +0.5 ‰ VSMOW) is used as an estimate of fractionation between seawater and coccolith calcite ($\alpha_{calcite\text{-}sw}$) to follow the palaeoceanographic usage. The $\delta^{18}O_{sw}$ values were measured in the work by Hermoso (2015) that used the same batch of seawater. The oxygen isotope composition of the inorganic calcite calculated using a recast formula of that given by Kim and O'Neil (1997) (equation 2).

$$\delta^{18}O_{inorg} = \frac{[\exp\left(\frac{18.03\times\frac{1000}{T+273.15}-32.17}{1000}\right)\times(\delta^{18}O_{sw}+1000)-1000]-30.91}{1.03091} \quad \text{(Eq. 2)}$$

where $\delta^{18}O_{inorg}$ is in ‰ VPDB; T is temperature on °C; pH is in total scale, and $\delta^{18}O_{sw}$ is in ‰ VSMOW.

3. Results

3.1. Change in cell size and growth rate with temperature

The specific growth rates (μ) in day^{-1} represent the exponential cell division rates, and are calculated after daily cell enumeration in the culture medium using a Coulter Counter apparatus. For all strains, μ increased with temperature in the range from 10 to 20 °C with the greatest variation between 10 and 15 °C (Fig. 2). In general, minimum and maximum growth rates were higher for *E. huxleyi* than for *G. oceanica*, with the exception of strain RCC 1314, which exhibited the highest measured μ at 20 °C (~ 1.5 day^{-1}). There was an overall maximum in μ at 20 °C except for *G. oceanica* RCC 1318, which is the only strain that divides fastest at 25 °C (Fig. 2). Maximum growth rates at 20 °C could reflect an ecological / physiological optimum related to a number of physiological parameters, such as the temperature-dependence of enzymatic activity, supply-to-demand ratio of carbon or other substrate necessary to build-up biomass and allow cell division. Other strains showed a growth rate inhibition at 25 °C relative to 20 °C. This was the case for *E. huxleyi* RCC 1212, and *G. oceanica* RCC 1211 and RCC 1314. The observed increase in μ with temperature up to 20 °C, followed by a decrease at higher temperatures is in agreement with the study by Langer et al. (2009) illustrating the great influence of temperature on cell division rates.

3.2. Change in oxygen isotope composition of coccolith calcite

The magnitude of oxygen isotope fractionation estimated by $\delta^{18}O_{calcite} - \delta^{18}O_{sw}$ indicates, for all strains and all growth temperatures, that the isotopic composition is heavier than predicted from inorganic conditions (Fig. 3). This is in agreement with all previously reported data and with assignment of species from this family to an isotopically "heavy group" for oxygen isotopes (Dudley et al., 1986; Ziveri et al., 2003; Rickaby et al., 2010; Hermoso, 2014; Hermoso et al., 2014; Stevenson et al., 2014; Hermoso et al., 2016; Rickaby et al., 2016). The three strains of *E. huxleyi* are more positively offset (i.e. have higher $\delta^{18}O$ values) than the three strains of *G. oceanica*. Other coccolith species precipitate calcite with lower $\delta^{18}O_{calcite}$ values relative to *E. huxleyi* and *G. oceanica* (Dudley et al., 1986; Hermoso, 2014). Coccoliths with near-equilibrium composition mainly include *Coccolithus pelagicus* and *Helicosphaera carteri* (Ziveri et al., 2003; Rickaby et al., 2010). All other coccoliths belonging to the isotopically "light group", such as *Calcidiscus leptoporus* and *Pleurochrysis carterae*, exhibit lower than equilibrium $\delta^{18}O_{calcite}$, values (Dudley et al., 1986; Ziveri et al., 2012; Candelier et al., 2013) (see Hermoso, 2014 for a review).

Over the entire range of growth temperature (10 – 25 °C), there is an overall statistically-significant linear correlation between $\delta^{18}O_{calcite} - \delta^{18}O_{sw}$ values and temperature ($r^2 > 0.92$). The 25 °C experiments depart from the oxygen isotope trend (slope) of the inorganic curve, as $\delta^{18}O_{calcite}$ values between 20 and 25 °C fail to record the predicted temperature component for a 5 °C change. For lower temperature experiments (10 and 20 °C), offsets between coccolith calcite $\delta^{18}O_{calcite}$ values at different temperatures indeed reflect those predicted by thermodynamics (Fig. 3).

Disregarding the 25 °C data points momentarily, data for the three colder temperature experiments display remarkably well behaved fits with temperature (mean $r^2 > 0.99$) (Fig. 3). However, the slopes that describe the 10 – 20 °C correlation between oxygen isotope fractionation with temperature are steeper than inorganic calcite for all examined species, suggesting that the magnitude of the vital effect decreases with increasing temperature. The isotopic composition of calcite produced at 25 °C can be compared with the trend seen between 10 and 20 °C (Fig. 3). By extrapolating the strong linear correlation in this range, we can infer a theoretical $\delta^{18}O_{calcite}$ at 25°C composition that records a 5°C subsequent warming step, as would be observed in inorganic (near equilibrium) calcite (Fig. 3). This calculation

allows us to infer an "excess" vital effect for experiments at 25 °C that can be quantified from 0.86 to +1.31 ‰ in $\delta^{18}O_{calcite}$ (mean for the six strains = +1.04 ‰).

Last, we observe that coccolith $\delta^{18}O$ data are significantly offset towards positive values with respect to the inorganic calcite curve established by Kim and O'Neil et al. (1997). With the exception of *G. oceanica* grown at 15 and 20 °C, we further note that all measured coccolith compositions are above the equilibrium limit of Watkins et al. (2013) (Fig. 3). In the present study, regardless of which equilibrium or inorganic calcite isotopic reference is chosen, we observe a consistent ^{18}O excess in measured Noelaerhabdaceae calcite $\delta^{18}O$ values.

3.3. Estimating the degree of utilisation of the internal carbon pool

Estimating the balance between the supply rate and utilisation of the *Ci*, hence assessing the dynamics of the carbon pool in the cell, can provide a constraint on the "residence time" of DIC, as this parameter has a foremost influence on the oxygen isotope system (Hermoso et al., 2014; Hermoso, 2015). A number of studies use the dynamics of carbon utilisation as a model to explain the carbon isotope fractionation in organic matter, including that of alkenones, by considering the combined effect of the supply rate via diffusion of ambient CO_2, together with the utilisation rate estimated by specific division rate (μ) and the cellular surface-area to volume ratio (Bidigare et al., 1997; Popp et al. 1998; Burkhardt et al. 1999; Popp et al. 2006; Riebesell et al., 2000; Laws et al. 2002; Pagani, 2002; Heureux et al., 2015). The data presented in Fig. 2 with calculation of $[CO_{2\ aq}]$ in media using temperature (see Methods) enables us to explore how the dynamics of carbon pool utilisation – *not only specific to the calcification pathways* – may also account for apparent oxygen isotopic fractionation in coccolith calcite, as is the case for the organic carbon isotope system (Bidigare et al., 1997). We see a significant linear correlation between the oxygen isotope composition and the "$\mu / [CO_{2\ aq}] \times$ volume / surface area" index after Bidigare et al. (1997). This index reflects the degree of internal carbon pool utilisation, and appears to scale with the oxygen isotope vital effect including the 25 °C experiments (Fig. 4).

We acknowledge that deriving the supply of carbon to the coccolithophore cell by using ambient $CO_{2\ aq}$ concentrations and cellular volume / surface area may be biased by the possibility of active or facilitated HCO_3^- acquisition by the cell (Nimer et al., 1999; Herfort et al., 2002; Kottmeier et al. 2014; Holtz et al., 2015). Under DIC limitation, it has been

quantified that ~ 10 % of the *Ci* may derive from HCO_3^- assimilation in some strains of *E. huxleyi* (Kottmeier et al. 2014). The intracellular transport of carbon from the cytosol to the coccolith vesicle is achieved in the form of HCO_3^-, but this latter intracellular phenomenon does not influence the "μ / $[CO_{2\,aq}]$ × volume / surface area" index *per se* since $CO_{2\,aq}$ is the dominant carbon source crossing the membrane from the external environment in marine phytoplankton.

4. Discussion

4.1. Coccolith $\delta^{18}O$ composition within an equilibrium / kinetic isotopic framework

Our isotope coccolith data can benefit from a comparison with recent work by Watkins et al. (2013, 2014) that allows a quantification of equilibrium versus kinetic limits in the $\delta^{18}O$ composition, hence offering a particularly relevant isotopic framework for understanding biologically-induced calcite precipitation. Indeed, Watkins et al. (2013; 2014) have documented an insightful reference for the interpretation of stable isotope compositions of biogenic and inorganic calcite, especially providing a reasonable envelope, which encompasses equilibrium and kinetic limits (Fig. 3). Within this isotopic framework, we see that the inorganic reference by Kim and O'Neil (1997) is close to the kinetic limit, hence calcite in their seminal work may not have been precipitated from an equilibrated DIC pool. This point suggests that the equation by Kim and O'Neil (1997) commonly used in palaeoceanography (Bemis et al., 1998; Ziveri et al., 2003; Candelier et al., 2013; Stevenson et al., 2014; Hermoso, 2015) does not strictly reflect equilibrium, as noted by Watkins et al., (2014). In the present dataset, most of the $\delta^{18}O$ values fall above any of these inorganic or computed isotopic references, including that of the "equilibrium limit" of Watkins et al. (2013). This important observation indicates that the expression of the vital effect in Noelaerhabdaceae coccoliths cannot be ascribed to a mere kinetic effect accompanying mineralisation, but is also the result of an isotopic disequilibrium in the H_2O – DIC system in the mineralising fluid prior to calcification. This point leads us to introduce the concept of an "^{18}O excess" in coccolith calcite, bearing in mind that this phenomenon is restricted to coccoliths belonging to the Noelaerhabdaceae Family (Dudley et al., 1986; Ziveri et al., 2003; Hermoso, 2014; Hermoso et al., 2016). Indeed, other coccolith species such as *Coccolithus*

pelagicus have been found with $\delta^{18}O$ values much more negative than *E. huxleyi* and *G. oceanica*, close to that of Kim and O'Neil (1997) values (Fig. 3). The restricted vital effect in *C. pelagicus* has been explained by slow dynamics of the *Ci* (Hermoso, 2014; Hermoso et al., 2014; Stevenson, 2014). The similarity in oxygen isotopic compositions between this coccolith and Kim and O'Neil's calcite indicate similar expression of kinetic effects in both inorganic and biogenic calcite. As such, the curve by Kim and O'Neil (1997) may represent a suitable "vital effect-free" reference for geochemical studies of coccoliths.

4.2. The isotopically "heavy group", a legacy of fast carbon fixation in phytoplankton?

Hermoso et al. (2014) and Hermoso (2015) recently hypothesised that the isotopically " heavy group" may be explained by a relatively high supply rate of isotopically heavy CO_2 to these cells, and the fast mineralisation of this internal *Ci* pool. One main difference between inorganically-precipitated calcite at equilibrium conditions and intracellularly-formed calcite in marine microalgae is the isotopic imprint of CO_2 in the internal *Ci* pool. This biogeochemical feature is due to the highly selective membranes of marine algae, that are much more permeable to CO_2 than to HCO_3^- (Giordano et al., 2005; Bach et al., 2013). Indeed, it is impossible to explain high $\delta^{18}O$ values in Noelaerhabdaceae coccoliths via the "equilibrium limit" of Watkins without invoking this – or *a* – source effect. As CO_2 has a very positive oxygen isotope signature compared to H_2O, CO_3^{2-} and HCO_3^- (~ +58; 40 and 24 ‰, respectively at 19°C after Zeebe and Wolf-Gladrow, 2001), the CO_2-rich flux into the *Ci* results in a relatively heavy oxygen isotopic composition of the DIC pool with respect to extracellular water. This disequilibrium can only be fleeting as re-equilibration of the H_2O – DIC system occurs in a matter of hours (Usdowski et al., 1991). The vital effect component is illustrated by the differences in the intercepts and slopes of the lines linking temperature and $\delta^{18}O_{coccolith}$, and temperature and $\delta^{18}O_{inorg}$ (Fig. 3), which in the case of the fast growing Noelaerhabdaceae coccoliths consists of the variable record of a ^{18}O-rich CO_2 source explained by non-equilibrium processes, especially operating at 25 °C.

The persistence of an isotopic disequilibrium in the oxygen isotope system between DIC acquired by the cell and CO_3^{2-} participating in calcification, and the resulting expression of a vital effect in calcite also indicate that a putative enzymatic activity by the carbonic anhydrase

in the cytosol does not fully re-establish equilibrium in Noelaerhabdaceae coccolith. Carbonic anhydrase, the enzyme that catalyses the inter-conversion of HCO_3^- and CO_2 with a large isotopic fractionation (Paneth and O'Leary, 1985; Uchikawa and Zeebe, 2012; Watkins et al., 2014) is either absent, or in insufficient quantity within the cell to achieve full oxygen isotope re-equilibration of the DIC system. To our best knowledge, this enzyme has only been found present in the periplasmic environment or associated with the chloroplast in coccolithophores (Quiroga and González, 1993; Nimer et al., 1994; Nimer and Merrett, 1996). The lack of identification of free cytosolic carbonic anhydrase in coccolithophores, or associated with the coccolith vesicle, renders a quantitative comparison of the present coccolith data with the biogeochemical work by Watkins et al. (2013, 2014) rather difficult. Hence, here we refrain from discussing the modulation of the ^{18}O vital effect based on the putative presence of carbonic anhydrase until further biological investigation is conducted on this topic.

4.3. Modulation of the oxygen isotope vital effect with temperature

The slopes of plots of temperature versus $\delta^{18}O_{calcite} - \delta^{18}O_{sw}$ are steeper for all strains from 10 to 20 °C than for all equilibrium / inorganic reference curves (Fig. 3). As coccolith $\delta^{18}O_{calcite}$ values tend to be closer to $\delta^{18}O_{inorg}$ with increasing temperature, this suggests a progressive decrease in the magnitude of the vital effect. A supplementary temperature control besides that dictating the kinetic fractionation between the DIC and calcite seems to operate in coccolith calcite. Further study is needed to determine the precise nature of this additional temperature control. Explaining lowered coccolith $\delta^{18}O$ values over the 10 to 20 °C temperature range by a modulation of the kinetic effect would require estimates for precipitation rates of calcite. From the experiments conducted by Sett et al. (2014), it can be deduced that in our own bioassays, temperature concomitantly induced increases in growth rate and calcification, the latter being estimated from the mass of inorganic carbon produced per cell in the study by Sett el al. (2014). Under the assumption that between 10 and 20 °C, calcite precipitated faster owing to increased temperature, we could hence explain steeper slopes in the relationship between temperature and $\delta^{18}O_{calcite} - \delta^{18}O_{sw}$ values compared to equilibrium values by an enhanced kinetic effect associated with changing precipitation rate (Fig. 3). However, it may be a gross over-simplification to assume that growth rate, or even bulk calcification scale with instantaneous precipitation rates, which is the only parameter relevant to a discussion of the modulation of kinetic effects.

Another possible mechanism for lower coccolith calcite $\delta^{18}O$ values is the so-called "carbonate ion effect" introduced for the coccolithophores (*Calcidiscus leptoporus*) by Ziveri et al. (2012). This explanation requires a supply of CO_3^{2-} ions to the cell, or alternatively higher pH at the site of calcification, which has been demonstrated in foraminifera (Spero et al., 1997). A recent study showed that in the coccolithophore *Gephyrocapsa oceanica*, lowered $\delta^{18}O$ at a given temperature was primarily due to enhanced CO_2 limitation and consecutive lower growth rates when the pH of the culture medium was increased, rather than the isotopic record of CO_3^{2-} (Hermoso, 2015). Carbonate ion assimilation in marine algae through a highly selective membrane remains an undemonstrated feature, that is only possible in foraminifera owing to vacuolisation of seawater as a source of ions for calcification. Furthermore, as we did not substantially alter CO_3^{2-} concentrations with changing temperature of the culture medium, such a hypothesis to explain our data can be dismissed.

Temperature exerts a number of effects on coccolithophores spanning from physiological to thermodynamic controls. Particularly relevant for understanding the oxygen isotope composition of coccolith calcite is the dynamics of the reactions leading to isotopic equilibrium between the oxygen atoms of water and DIC before calcification occurs (Usdowski et al., 1991; Zeebe and Wolf-Gladrow, 2001; Beck et al., 2005; Zeebe, 2007; Fig. 5). The duration of the formation of one single coccolith of *E. huxleyi* during the light period is approximately two hours (Westbroeck et al., 1989). During this period, cells continuously assimilate DIC (HCO_3^-) into the coccolith vesicle to build the coccolith. The time needed to erase the ^{18}O-CO_2-induced isotopic disequilibrium is on the order of 12 h at 15 °C (Usdowski et al., 1991; Zeebe and Wolf-Gladrow, 2001). This means that the isotopic disequilibrium introduced to the *Ci* by assimilation of CO_2 takes longer than calcification, and also, that this disequilibrium is erased more completely at 20 °C than at 10 °C (Fig. 5). Indeed, over this temperature range, the magnitude of the vital effect becomes linearly halved using the calibration line of Kim and O'Neil (1997) as a reference (Fig. 3). This feature may explain a lower apparent vital effect (sensu the departure of $\delta^{18}O_{calcite}$ from inorganic calcite) with increase in temperature. Furthermore, this process is able to account for maximum $\Delta\delta^{18}O$ measured between 10 and 15°C, as the gradient of re-equilibration time is larger at low temperature (Fig. 4).

Perhaps, the most intriguing feature in this dataset is the lack of a clear isotopic change in $\delta^{18}O$ values between 20 and 25 °C (Fig. 3). If a linear increase in growth rates with decrease in the magnitude of the vital effect is observed between 10 and 20 °C, this contrasts with

observations made for 25°C, the temperature at which cell growth becomes slower, as a likely result of $CO_{2\ aq}$ limitation (ambient $[CO_{2\ aq}] < 10$ µmol C kg_{sw}^{-1}). At this temperature, cell division is still high, but on the same order, or less than for 20 °C, depending on the strain considered.

At 25 °C, ambient CO_2 concentrations dissolved in the medium are lower than in colder temperatures, but the supply rate of ^{18}O-rich influx from CO_2 assimilation becomes larger as it is not matched by an increase in utilisation. This decoupling between the carbon resource and the demand-to-supply ratio lead to a greater ^{18}O imprint of the *Ci*, and hence, enhanced magnitude of the vital effect – a feature recently revealed for *Gephyrocapsa* sp. coccoliths over glacial –interglacial cycles of the Pleistocene in the Caribbean Sea (Hermoso (2016). This mechanism can explain why the oxygen isotopic composition of calcite produced at 25 °C is 1 ‰ more positive than extrapolated values only if the thermodynamic re-equilibration process of the *Ci* between 10 and 20 °C is at play (Fig. 3). Overall, we show here how the dynamics of the intracellular carbon cycle exerts a strong control on the oxygen isotopic composition of coccoliths, superimposing a biological (vital) signal on the temperature-dependence of $^{18}O/^{16}O$ fractionation in calcite.

4.4. Geological implications for reconstruction of sea surface temperature using coccolith-dominated sediments

As it is now possible to concentrate coccolith fractions from sediments (e.g. Minoletti et al., 2009), understanding the departure of carbon and oxygen isotopes from equilibrium conditions and constraining the driving mechanism of the vital effect is essential to refine palaeoenvironmental information relying on coccolith biominerals.

Overall, we demonstrate that the use of oxygen isotope compositions of Noelaerhabdaceae coccoliths in palaeoceanography to derive sea surface temperatures is possible, but requires other constraints on the growth of algae that can be estimated from emerging palaeo-cell size proxies. Extant coccolithophore species belonging to the Noelaerhabdaceae are characterised by relatively small cell size compared to their geological ancestors. This feature results from a long-term reduction in cell size of coccolithophores of this taxon over the Cenozoic that was likely induced by long-term declining pCO_2 (Henderiks and Pagani, 2008) and optimisation of CO_2 sourcing by the cells. Over a long time scale, increased surface-area to volume ratios

alleviated CO_2 limitation by increasing the supply rate of carbon via passive CO_2 diffusion (Francois et al., 1993; Bolton and Stoll, 2013; Holtz et al., 2015). This feature may be accompanied by evolution of the ^{18}O vital effect within the Noelaerhabdaceae lineage, as apparent over glacial/interglacial timescales and during the Neogene (Bolton and Stoll, 2013; Hermoso, 2016).

The coccolithophores also produce another valuable palaeo-proxy archive the alkenones (Prahl et al., 2000). It is possible, by analysing the degree of undersaturation of these lipids ($U^{k'}_{37}$) to derive seawater temperatures (Prahl et al., 2000; Conte et al., 2006). Hence the residual between $U^{k'}_{37}$ and $\delta^{18}O_{calcite}$-derived temperature estimate has the potential to bear palaeoenvironmental and physiological information related to the carbon cycle at the cellular level over a time interval of the Neogene. Such a biogeochemical development with a palaeoceanographic aim can be made by undertaking an intergroup (planktonic foraminifera and coccoliths) isotopic approach.

Culture conditions and natural environmental settings differ in many ways, such as the replete conditions imposed in the laboratory (concentrations in nitrate, phosphate, vitamins, trace metals, appreciable light intensity, optimal CO_2 levels) and the absence of ecological competition for these resources. As such, transferring the culture data of the present study to calcareous nannofossils in a downcore study may be flawed without integrating a "CO_2 concentration context" (Hermoso et al., 2015). During greenhouse periods with SSTs exceeding 25 °C, it cannot be concluded from our study that the oxygen isotope vital effect was significant, as elevated temperatures are due primarily to very high CO_2 levels. Indeed, in high CO_2 / high temperature oceans, the coccolith vital effects were probably minimum owing to alleviated carbon limitation for algae (Hermoso et al., 2016). Other environmental factors at play in the ocean may have a secondary influence on any parameters governing growth, size of the *Ci* and oxygen isotope composition of calcite (such as pH and $[CO_3^{2-}]$). Reproduction of more accurate and true oceanic conditions in the laboratory needs to be achieved in order to gain a more geologically-relevant picture of the modulation of the vital effect in coccolithophores.

5. Conclusions

This culture study shows that the magnitude of the vital effect affecting the oxygen isotope composition of cultured coccolith calcite is not constant, and is primarily driven by the dynamics of the internal carbon pool from assimilation to mineralisation, which is controlled by temperature. When temperature and growth rates are linearly correlated, the magnitude of the vital effect consisting of the record of a ^{18}O-rich pool inherited from partial CO_2 assimilation by the cell is nearly constant. There is, however, a slight decrease in the magnitude of this vital effect with increasing temperature, as this isotopic disequilibrium is more extensively erased with increasing temperature. In contrast, the vital effect is exacerbated when the coccolithophores face CO_2 limitation, as is the case at 25 °C in our experiments. Overall, this study confirms the "*mobilis in mobili*" nature of the vital effect illustrating the changing intensity of this biological phenomenon under changing environmental conditions (Hermoso, 2014), but demonstrates that its magnitude in Noelaerhabdaceae coccoliths is set by the supply-to-utilisation balance of the internal DIC pool.

By dissecting the vital effect, our study shows that temperature has three distinct influences on oxygen isotope composition of coccolith calcite: *i*) control of solubility of aqueous CO_2 in seawater; *ii*) control on division rate, cell size and residence time of carbon in the *Ci* pool; and ultimately *iii*) control on fractionation between DIC and calcite at times of intracellular calcification. It is worth nothing that only *ii*) can be assigned to a vital effect component. Decoupling the relative importance of these processes at different steps between assimilation and mineralisation of DIC can be achieved via biogeochemical modelling in the future.

Acknowledgements

This work was supported through European Research Council (ERC) grant SP2-GA-2008-200915 awarded to R.E.M. R. M. H. and R.E.M. R. are also grateful to NERC Ocean Acidification Programme for additional funding (grant NE/HO17119/1). FM thanks the CNRS for financial support from a grant SYSTER with reference AO2014 – 884402. Coccolithophore strains cultured at Oxford were provided by the Roscoff Culture Collection via the EU FP7 Research Infrastructure Initiative "ASSEMBLE" Ref. 227799 to M.H. The authors are grateful to Ruth Blake for editorial handling of the paper and to Emanuela Mattioli and two anonymous referees for their constructive reviews. This is IPGP contribution

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Figures

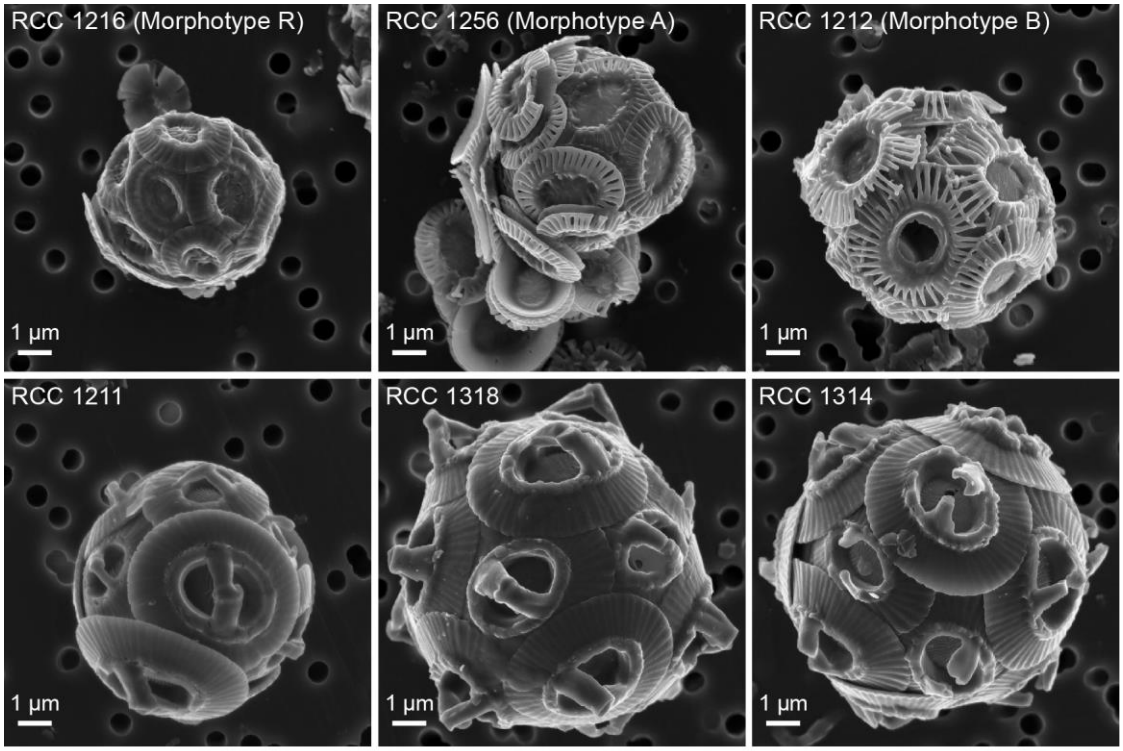

Figure 1. Scanning electron microscope images of coccospheres produced by coccolithophore species of the Noelaerhabdaceae Family cultured in the present study. Top row: *Emiliania huxleyi*. Bottom row: *Gephyrocapsa oceanica*. Roscoff Culture Collection (RCC) strain numbers, scale bars and morphotypes for *E. huxleyi* are inset.

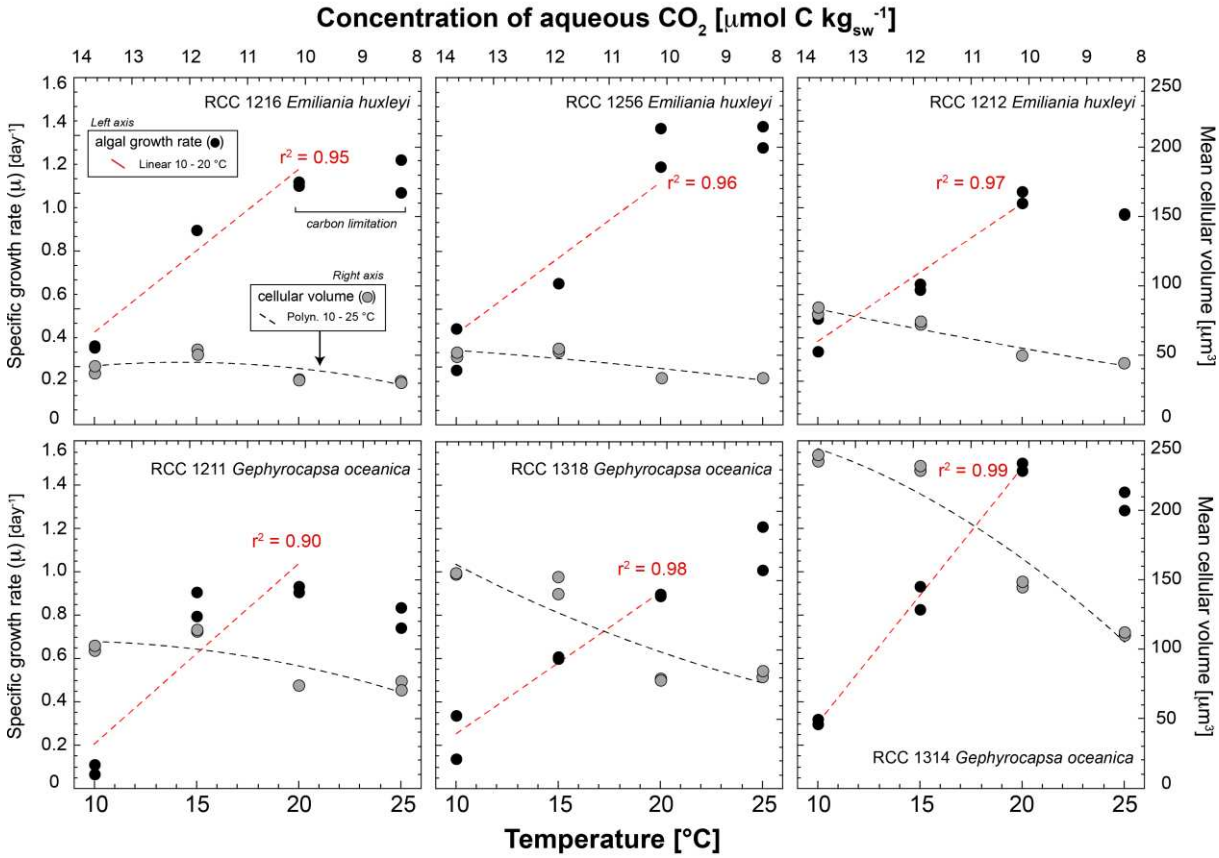

Figure 2. Specific growth rates (filled dots - left *y*-axis) and cellular volume (open circles - the right *y*-axis) against temperature for the six strains of Noelaerhabdaceae coccolithophores. Concentrations of aqueous CO_2 as a function of growth medium temperature are indicated (top of *x*-axis). The two points at a given temperature correspond to culture replicates. There is a clear linear increase in the division rates (µ) over the temperature range 10 – 20 °C with a more pronounced increase between 10 and 15 °C. At 25 °C, cells do not show enhanced division rate with respect to 10 to 20 °C range. Strains RCC 1212; 1211 and 1314 exhibit inhibited growth over 20 °C. We observe an overall progressive decrease in cell volume (corresponding to an increase in their surface area to volume ratios over the 10 – 25 °C range).

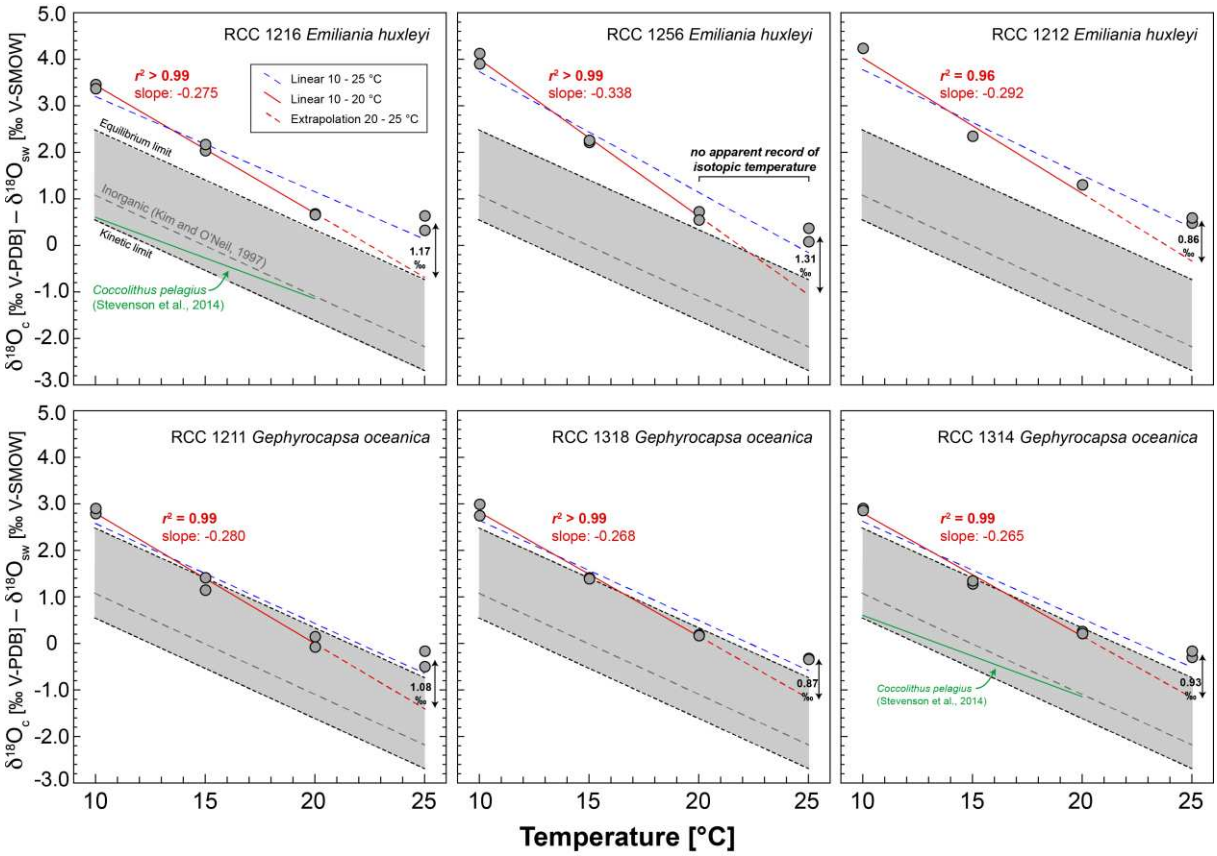

Figure 3. Change in the apparent oxygen isotope fractionation of coccolith calcite with temperature. We observe a linear relationship between 10 and 25 °C (thin red dashed lines), approximately parallel to inorganic calcite, with *Emiliania huxleyi* being more positively offset than *Gephyrocapsa oceanica*. This relationship is clearest between 10 and 20 °C (plain red lines). These steeper slopes compared to the inorganic calcite calibration indicate that the magnitude of the vital effect decreases with increasing temperatures, as a result of faster thermodynamic re-equilibration of isotopes between the internal carbon pool and water with temperature. Experiments at 25 °C show exacerbated magnitude of the vital effect compared the 10 – 20 °C linear trend. The double arrows and isotopic offsets reported below the 25 °C datapoints are the isotopic offsets between the extrapolated values from the 10 - 20 °C trend and the mean of the two measured replicates at 25 °C. The magnitude of these offsets represents the vital effect component imparted to CO_2 limitation by the cells. The grey area denotes the range of equilibrium – kinetic compositions of calcite reported from the work by Watkins et al. (2013).

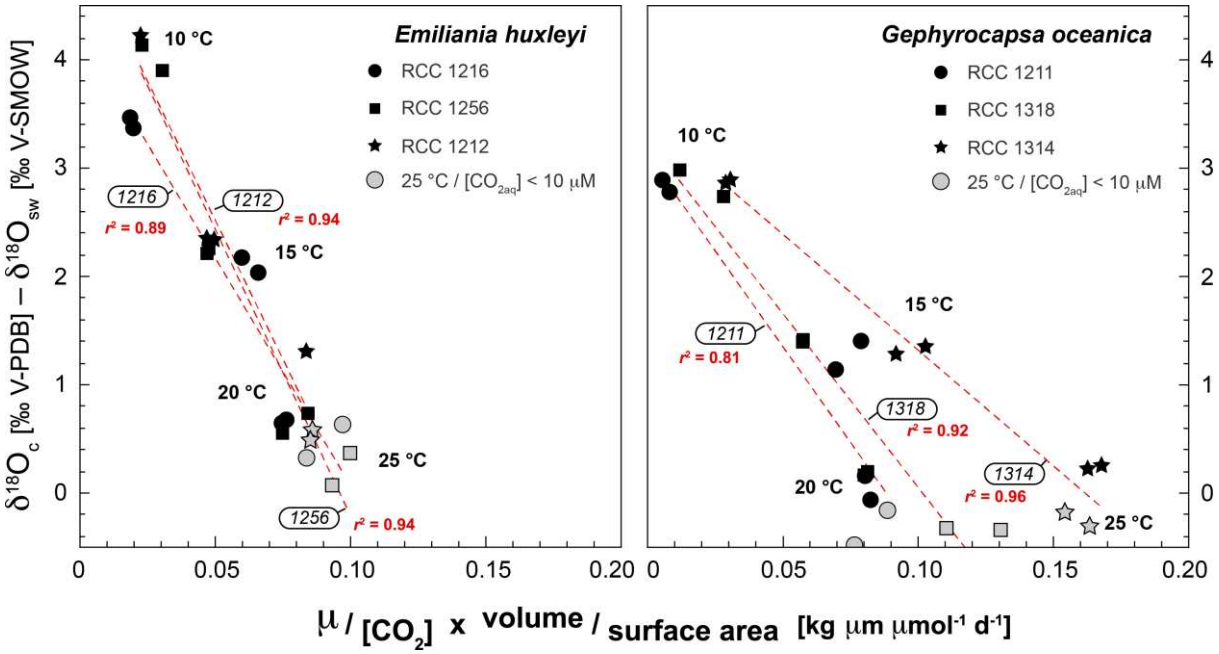

Figure 4. Change in the magnitude of the apparent oxygen isotope fractionation ($\delta^{18}O_{calcite} - \delta^{18}O_{equilibrium}$) with the degree of utilisation of the carbon pool in cultured algae (as estimated by the index "μ / $[CO_2]$ × volume / surface area"). We see statistically-significant linear relationships between departure of inorganic (vital effect) and this index, indicating that the dynamics of the intracellular carbon cycle (demand-to-supply ratio) sets the magnitude of the oxygen isotope vital effect in fast growing coccolith calcite.

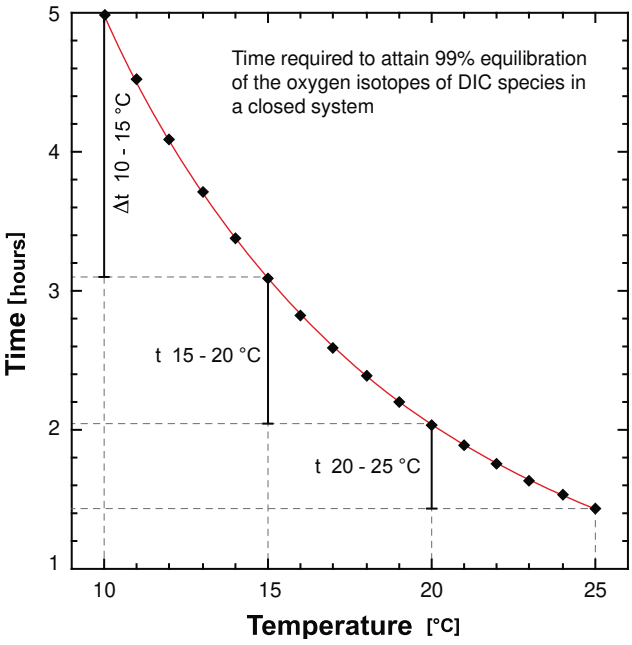

Figure 5. Time required to reach 99 % of isotopic re-equilibration in the oxygen system in solution for a closed system. Calculations have been made according the formula given by Usdowski et al. (1991) for a pH 7. The red line denotes an exponential fit of calculations made for each temperature over the 10 – 25 °C range. We see that the 5 °C increments (Δt), as implemented in our laboratory culture strategy, progressively represent shorter re-equilibration times. For reference, the duration of the formation of one individual coccolith of *E. huxleyi* is about 2 hours (Westbroeck et al., 1989).