Vanishing coccolith vital effects with alleviated carbon limitation

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Abstract

By recreating a range of geologically relevant concentrations of dissolved inorganic carbon (DIC) in the laboratory, we demonstrate that the magnitude of the vital effects in both carbon and oxygen isotopes of coccolith calcite of multiple species relates to ambient DIC concentration. Under high DIC levels, all the examined coccoliths exhibit significantly reduced isotopic offsets from inorganic calcite compared to the substantial vital effects expressed at low (preindustrial and present-day) DIC concentrations. The supply of carbon to the cell exerts a primary control on biological fractionation in coccolith calcite via the modulation of coccolithophore growth rate, cell size and carbon utilisation by photosynthesis and calcification, altogether accounting for the observed interspecific differences between coccolith species. These laboratory observations support the recent hypothesis from field observations that the appearance of interspecific vital effect in coccolithophores coincides with the long-term Neogene decline of atmospheric CO_2 concentrations and bring further valuable constraints by demonstrating a convergence of all examined species towards inorganic values at high pCO_2 regimes. This study provides palaeoceanographers with a biogeochemical framework that can be utilised to further develop the use of calcareous nannofossils in palaeoceanography to derive sea surface temperature and pCO_2 levels, especially during periods of relatively elevated pCO_2 concentrations, as they prevailed during most of the Meso-Cenozoic.

1 Introduction

The quest to generate reliable and accurate palaeoenvironmental reconstructions is hindered by uncertainties in our current proxies from the sedimentary archive. One prominent caveat owes to the biological origin of sedimentary calcareous particles in marine and oceanic realms. As a consequence of the biological controls on chemical signals in algae, most biominerals do not precipitate at equilibrium conditions and the compositional departure between biocarbonates and an inorganic reference is commonly referred to as the vital effect. Therefore, geochemical data from ancient biomineralising organisms must be corrected in order to derive the primary signals from palaeoseawater. In the case of the foraminifera, corals and coccoliths, the foremost carbonate producers in the marine realm, there has been a considerable number of studies during which living organisms were cultured in strictly controlled environmental conditions and their biominerals measured for a range of isotopic systems to generate empirical proxy calibrations (Erez and Boa, 1982; Dudley et al., 1986; Spero et al., 1997; Bemis et al., 1998; Ziveri et al., 2003; Tripati et al., 2010; Rickaby et al., 2010; Rollion-Bard et al., 2011; Grauel et al., 2013; Hermoso et al., 2014; Minoletti et al., 2014; Hermoso, 2015).

Another important aim in palaeoceanography is to determine whether the physiology-induced fractionation for a given taxon was constant through time from an evolutionary perspective, and over shorter time intervals comprising large climatic fluctuations, in turn inducing an environmentally-driven modulation of the vital effect (Hermoso, 2014). In the absence of more reliable information, the Uniformitarianism principle – by which, the processes that were operating in the geological past still exist today, and *vice-versa*, is commonly applied for elucidating vital effects and reconstructing primary oceanographic signals.

Although coccoliths are relatively challenging to extract at the species-specific level from sediments compared to foraminifera, coccolith-based studies represent a growing field since the pioneering work by Anderson and Steinmetz (1981). To better interpret coccolith isotope signals and generate more reliable palaeoenvironmental estimates from these cosmopolitan organisms, we need to gain a broader picture of their vital effects, and more specifically determine how environmental parameters govern their magnitude. Several studies have specifically measured coccolith $\delta^{18}O$ with changing temperature in laboratory cultures in order to determine and calibrate the temperature / $\delta^{18}O$ relationship for a wide range of species (Dudley et al., 1986; Ziveri et al., 2003; Candelier et al., 2013; Stevenson et al., 2014). Meanwhile, other culture studies have kept temperature constant but have manipulated the carbonate chemistry of the culture medium and the irradiance level (Ziveri et al., 2003; Rickaby et al., 2010; Hermoso, 2015) and found substantial modulation of the oxygen isotope vital effect with these parameters at constant temperature. In most cases, only one parameter was controlled at a time, and we are lacking cross-parameter investigations that are required for the effective application of palaeoproxies. In nature, environmental parameters generally co-vary, such as sea surface temperatures and pCO_2 concentrations. This is illustrated by the recent natural environment study by Hermoso et al. (2015) analysing coccoliths microseparated from core top sediments, which further illustrates the intricate (multi-parameter) control of coccolith oxygen and carbon isotope compositions ($\delta^{18}O$ and $\delta^{13}C$, respectively).

These biogeochemical proxies raise questions regarding what vital effect coefficients should be applied to ancient coccolith species extracted from Meso-Cenozoic sediments as temperature and pCO_2 significantly evolved before reaching preindustrial levels. In the present study, we document a multi-species control of stable carbon and oxygen isotope composition under a wide range of DIC (hence pCO_2) levels at a constant pH of 8.2 (total scale) recreated in the laboratory. As varying the availability of ambient DIC substrate may modulate the degree of carbon limitation for algal growth (cell division rate and size), this culture approach will allow us to determine whether the vital effect is constant for a given coccolith species or changes with the environment, and in particular in response to ambient carbon concentrations.

2 Material and methods

2.1 Coccolithophore strains studied

Emiliania huxleyi has attracted most recent attention in coccolithophore research due to its dominance in present-day oceans, its importance in biogeochemical cycles, and accompanying relevance to ocean chemistry and climate of the Anthropocene (*e.g.*, Bidigare et al., 1997; Riebesell et al., 2000; Iglesias-Rodriguez et al., 2008; De Bodt et al., 2010; Suffrian et al., 2011; Müller et al., 2012; Bach et al., 2013; Sett et al., 2014; Tchernov et al., 2014; Young et al., 2014; Aloisi, 2015; Holtz et al., 2015). The strain RCC 1256 used in this study produces lightly calcified coccoliths assigned to the morphotype A (Langer et al., 2011). From a geological point of view however, palaeoceanographic applications on *E.*

huxleyi only cover a narrow time interval as this species has only recently evolved (~ 268 kyr ago; Thierstein et al., 1977).

The species *Calcidiscus leptoporus* has a longer geological record with its first appearance in pelagic sediments reported in the Miocene (Bown, 1998). This species was studied in culture to assess changes in the morphology of its coccoliths with altered medium chemistry (Langer and Bode, 2011) and isotopically (Ziveri et al., 2012; Candelier et al., 2013; Hermoso et al. 2014). In the present study, we used the strain RCC 1129 corresponding to the intermediate morphotype on the merit of coccolith size. The same monoclonal strain was previously cultured by Candelier et al. (2013) and Hermoso et al. (2014) where cells were successively subjected to change in temperature and medium oxygen composition.

The large and relatively ancient taxon *Coccolithus pelagicus* (strain RCC 1202 being studied here) corresponds to the subspecies *braarudii*. This taxon has been examined isotopically in culture (Rickaby et al., 2010; Hermoso et al. 2014; Stevenson et al., 2014). Amongst all extant coccolithophore species, *C. pelagicus* has the longest geological record with a first occurrence of the informally defined "*C. pelagicus* group" dated back to the Palaeogene (~ 66 Myr ago).

Pleurochrysis placolithoides has no direct geological relevance. The occurrence of this species has not been reported in the fossil record owing to its nearshore ecology compared to most coccolithophore species living in more open ocean settings (Young et al., 2003). However, its coccosphere size is in between *C. pelagicus* and *C. leptoporus* – all taxa belonging to the Coccolithales Order. As these two strains have contrasting vital effects, it is interesting to study an intermediate cell size to further explore a link between cell morphology and coccolith isotopic composition. The strain used in this study is RCC 1401.

2.2 Culture medium preparation

A raw batch of natural seawater collected from the English Channel (Station L4; 50° 15.00' N – 4° 13.02' W) was supplied by MBA, Plymouth (UK). The batch of seawater (salinity ~ 33) was first acidified using concentrated HCl to reach pH ~ 2, conditions under which most of the dissolved inorganic carbon was present in form of aqueous CO_2. The batch was bubbled overnight with pure N_2 to remove DIC. Subsequently, pH was brought back to a value around 8 by addition of NaOH. Still under N_2 purge, we amended the medium in nitrate, phosphate, EDTA and vitamins according to the *K/2* recipe (see Hermoso et al., 2014 for further details). To obtain the desired DIC level (2; 4; 6; 8; 10 and 12 mmol / kg_{sw}), we proceeded to add calculated amounts of $NaHCO_3$ powder (Sigma – Batch CAS 144-55-8) in different aliquots with immediate pH adjustment to 8.2 (total scale), after which each DIC batch then was promptly filtered-sterilised and kept in Teflon-sealed flasks without headspace. Prior to inoculation, each medium was measured for its total alkalinity using a 916 Ti Touch automatic titrator (Metrohm) (Table 1). Successive alterations of the carbonate chemistry, due to the addition of HCl, $NaHCO_3$ and NaOH, did not induce change in total alkalinity compared to the original seawater batch, and there was a very good agreement between target and measured DIC concentrations for each batch (within a range of 5 %).

2.3 Cell density, size and growth

During the acclimation and culture phases, cells were maintained at 15 °C and illuminated under a daily 14h/10h light/dark cycle in Sanyo MLR-351 plant growth chambers. The irradiance was measured as 150 µmol photons m^{-2} s^{-1}. Duplicate culture batches were performed semi-continuously to allow DIC to remain stable with cell growth and preferential CO_2 assimilation and utilisation by the cells leading to increasing pH (Hermoso, 2014), which conforms to experimental guidelines (Barry et al., 2010). Unfortunately due to this experimental set-up, too low amount (mass) of harvested culture residues has prevented us from generating meaningful PIC/POC ratios for this study.

The evolution of culture growth was determined by cell enumeration made every two days, approximately 3 hours after the onset of the illuminated phase using a Beckman Coulter Counter Series Z2 apparatus fitted with a 100 µm aperture tube. The diluent used was ISOTON II obtained from Beckman Ltd. Calibration of spherical-equivalent coccosphere sizes was performed daily using 10.16 µm diameter latex beads provided by the same company. Coccosphere sizes were determined by the mode of Gaussian distribution on the coccospheres given by the Coulter Counter device (Table 1). The specific growth rates (µ) were calculated from cell densities measured at time of culture harvest (c_f) and two days prior to that (c_{f-2}), using the formula:

$$\mu\ [day^{-1}] = [\ \ln(c_f) - \ln(c_{f-2})\ ] / 2 \qquad (1)$$

where cell concentrations are in number of cells per mL of culture medium.

2.4 Isotopic analyses

Carbon and oxygen isotope compositions of coccolith calcite and the oxygen isotopic ratios from water media were measured as described in Hermoso et al. (2014). In brief, coccolith calcite from rinsed and oxidised culture residues were measured using a VG Isogas Prism II mass spectrometer with an on-line VG Isocarb at Oxford University. Results ($\delta^{18}O_c$ and $\delta^{13}C_c$) are expressed against the international V-PDB reference (Table 1). Medium $\delta^{18}O$ compositions ($\delta^{18}O_{sw}$) were determined by gas-water exchange on a Delta Gas Bench II coupled to a Delta V Advantage mass spectrometer at the University of Oxford. A similar value was obtained for all the DIC batches with a typical value of +0.50 ‰ V-SMOW.

The $\delta^{13}C$ of $NaHCO_3$ powder used was directly measured on the Prism with a value of −2.54 ‰ V-PDB. Subsequently, $\delta^{13}C$ of DIC ($\delta^{13}C_{DIC}$) were measured at Cambridge University using a Thermo Gas Bench attached to a Delta V mass spectrometer and isotopic values were similar for each batch (within typical error of ± 0.1 ‰) and indistinguishable from that of the $NaHCO_3$ powder employed to amend the growth milieus.

The magnitude of the vital effects for the oxygen and carbon isotope systems is expressed as the isotopic offset of coccolith calcite from inorganic calcite ($\delta^{18}O_{inorg\ and}$ $\delta^{13}C_{inorg,}$, respectively) calculated using the equations provided by Kim and O'Neil (1997) and Romanek et al. (1992).

$$^{18}O \text{ Vital effect [‰ V-PDB]} = \delta^{18}O_c \text{ [‰ V-PDB]} - \delta^{18}O_{inorg} \text{ [‰ V-PDB]} \qquad (2)$$

where $\delta^{18}O_{inorg}$ is calculated after the equation of Kim and O'Neil (1997) and Bemis et al. (1998). We note that this computed oxygen isotopic composition is indistinguishable from that of Watkins et al. (2014) with a biogenic-relevant kinetic effect (see Hermoso, 2015). For our experiments, a constant $\delta^{18}O_{inorg}$ value of −0.04 ‰ V-PDB was calculated for a temperature of 15 °C and an oxygen isotope composition of the culture medium of + 0.5 ‰ V-SMOW.

$$^{13}C \text{ Vital effect [‰ V-PDB]} = \delta^{13}C_c \text{ [‰ V-PDB]} - \delta^{13}C_{inorg} \text{ [‰ V-PDB]} \qquad (3)$$

where $\delta^{13}C_{inorg}$ is calculated as $\delta^{13}C_{DIC}$ +1 (Romanek et al., 1992), hence $\delta^{13}C_{inorg}$ has a constant value of +1 ‰ expressed in the $\delta^{13}C_c - \delta^{13}C_{DIC}$ referential.

3 Results

3.1 Modern-day dissolved inorganic carbon concentration (~ 2 mmol / kg of DIC)

3.1.1 Growth rates and coccosphere sizes

Cell division rates at pre-industrial DIC levels (~ 2 mmol / kg) are similar to those found in published literature (Langer et al., 2006, 2009; Rickaby et al., 2010; Bach et al., 2013; Candelier et al., 2013; Hermoso et al., 2014; Kottmeier et al., 2014; Sett et al., 2014). *Emiliania huxleyi* is the fastest grower for the smaller cell size, achieving about one division per day (μ ~ 0.7 day^{-1}) (Fig. 1a; Fig. 1b). The largest cells of *C. pelagicus* and *P. placolithoides* (19 and 16 µm diameter on average, respectively) show specific growth rates around 0.5 day^{-1}. *Calcidiscus leptoporus* with a coccosphere diameter between *E. huxleyi* and *P. placolithoides* (~ 10 µm) exhibits the lowest division rates among all examined species at 2 mmol / kg of DIC, with μ values around 0.3 day^{-1} (Fig. 1a; Fig. 1b).

3.1.2 Carbon isotope composition of coccolith calcite

The interspecies range in coccolith $\delta^{13}C_c$ values grown under preindustrial CO_2 levels (~ 270 ppm / ~ 2 mmol / kg of DIC) is considerable, on the order of 3 ‰ (Fig. 2a). This variation confirms the presence of very large vital effects for the carbon isotope system (Ziveri et al., 2003; Rickaby et al., 2010; Hermoso et al., 2014). Coccolith calcite carbon isotopic compositions are distributed either side of the inorganic reference value (Fig. 2a): *E. huxleyi* and *P. placolithoides* exhibit positive $\delta^{13}C$ values (hence, a "positive" ^{13}C vital effect). Due to insufficient calcite yield at harvest for isotopic analysis for *P. placolithoides* grown at 2 mmol / kg of DIC, the assignment of *P. placolithoides* to an isotopic "heavy group" (*sensu* Dudley et al., 1986) is inferred by extrapolation from the 4 – 12 mmol / kg range. *C. pelagicus* and *C. leptoporus* meanwhile have relatively similar $\delta^{13}C_c - \delta^{13}C_{inorg}$ values, corresponding to a −2.5 ‰ vital effect. Overall, these numbers are in good agreement with published literature when cultures were grown at low cell concentration (see synthesis in Hermoso, 2014). However, substantial differences in the carbon isotope fractionation of *E. huxleyi* are apparent between the data reported here and in the work by Rost et al. (2002) (Fig. 2a). It is worth noting that different methodologies were applied to manipulate the carbonate chemistry of the media. In the present study, [DIC] and [$CO_{2\,aq}$] linearly covary, whereas in the work by Rost et al. (2002), [DIC] was constant, but the pH values of each batch were different (7.9 – 8.6), hence suggesting a combined CO_2 availabity and pH effect on coccolith $\delta^{13}C$ values. The same study also revealed a modulation of the ^{13}C vital effect operated by the intensity of light irradiance and by the photoperiod.

3.1.3 Oxygen isotope composition of coccolith calcite

The $\delta^{18}O$ of coccolith calcite grown by algae exposed to 2 mmol / kg of DIC is also comparable to values reported in literature with media aerated with laboratory air (Ziveri et al., 2003; Candelier et al., 2013; Hermoso et al., 2014; Stevenson et al., 2014) (Fig. 2b). Our data are thus compatible with the assignment of coccolith species into three groups on the merit of oxygen isotope composition either from $\delta^{18}O_c - \delta^{18}O_{sw}$ or from $\delta^{18}O_c - \delta^{18}O_{inorg}$ values (the latter being used to quantify the magnitude of the "vital effect"; Eq. 2). *Emiliania huxleyi* ("heavy group") has the most positive $\delta^{18}O_c$ values and large vital effects (+2 ‰) (Fig. 2b). *Coccolithus pelagicus* ("equilibrium group") produces calcite with oxygen isotope composition close to that of inorganic calcite, although in the present study, the values are slightly (~ 0.5 ‰) shifted towards relatively high $\delta^{18}O$ ratios. *Calcidiscus leptoporus* ("light group") exhibits lower $\delta^{18}O_c$ values than the inorganic reference (Fig. 2b). The offset from inorganic calcite is −1.4 ‰ for *C. leptoporus*, the same magnitude of the vital effect reported by Candelier et al. (2013) rather than those by Dudley et al. (1986). By extrapolation from higher DIC levels in amended medium, it can be deduced that *P. placolithoides* would belong to the "light group", which is consistent with the work of Dudley et al. (1986) concerning the closely related species *Pleurochrysis carterae.*

3.2 Effect of increased DIC (at constant pH) on growth and isotopes (4 – 12 mmol / kg of DIC)

3.2.1 Change in cell size and growth rate with increased DIC

Contrasting responses among examined species are observed in the evolution of specific growth rates and coccosphere volume with increased ambient DIC level, and as a result, in the carbon resource around the cells (Fig. 1a; Fig. 1b). The relatively fast growing *E. huxleyi* species exhibits fertilisation (higher growth rates) from 2 to 8 mmol / kg, beyond which a decrease is observed at the highest DIC levels. A similar decrease at high alkalinity was previously observed on the close relative *Gephyrocapsa oceanica* (Rickaby et al., 2010). Both

C. leptoporus and *C. pelagicus* decreased cellular division rates over the 2 to 12 mmol / kg range of DIC concentration, but decreased growth rates are marked for *C. pelagicus* with μ linearly changing from 0.5 down to 0.1 day^{-1} with increasing DIC concentrations. Changing ambient DIC does not induce significant modulation of growth rate for the species *P. placolithoides*. One may expect decreased μ to be accompanied by longer generation time, and hence larger cell sizes (Aloisi, 2015). However, there is no covariation between growth rates and coccosphere and cell sizes for the species examined here (Fig. 1a; Fig. 1b). Nevertheless, the data confirm that both *E. huxleyi* and *P. placolithoides* cells become relatively larger with elevated DIC levels, as observed for the former in the work by Müller et al. (2012). *Calcidiscus leptoporus* exhibits no change in size with DIC availabity, whereas *C. pelagicus* shows significantly decreased coccospheres sizes at high DIC levels.

3.2.2 Change in carbon isotope composition of coccolith calcite

With increased DIC concentration in the culture medium, species that exhibited high $\delta^{13}C$ values at 2 mmol / kg of DIC show a significant decrease in $\delta^{13}C_c - \delta^{13}C_{DIC}$ values, hence a diminished vital effect (Fig. 2a). The observed decreases in $\delta^{13}C_c - \delta^{13}C_{DIC}$ with increasing DIC are linear ($r^2 = 0.96$ for *E. huxleyi* and 0.70 for *P. placolithoides*). At the highest DIC concentrations, it appears that the averages between the two duplicates show coccolith calcite $\delta^{13}C_c$ values for these two species indistinguishable from that of the inorganic reference (*sensu* Romanek et al., 1992), hence vital effects vanish at high DIC. By contrast, species with lowest $\delta^{13}C$ at 2 mmol / kg (*C. pelagicus and C. leptoporus*) show increased carbon isotope compositions with addition of DIC in the medium, a trend that also corresponds to a strong decrease in the expression of the vital effect for these species (Fig. 2a). This positive evolution is linear for *C. leptoporus* ($r^2 = 0.83$) and *C. pelagicus* ($r^2 = 0.85$), although for the latter largest species the 2mmol / kg datapoints departs from the 4 – 12 mmol / kg linear trend with substantial low $\delta^{13}C$ values. This "jump" in *C. pelagicus* $\delta^{13}C$ values between 2 and 4 mmol / kg represents most of the evolution in the $\delta^{13}C$ composition over the whole range of DIC concentration investigated here. At the highest DIC concentration, *C. pelagicus* exhibits near inorganic $\delta^{13}C$ values, whereas *C. leptoporus* remains –0.4 ‰ negatively shifted from this reference.

3.2.3 Change in oxygen isotope composition of coccolith calcite

The typology of a heavy and light isotopic group for the oxygen isotope system still exists with increased ambient DIC concentration, but the magnitude of the vital effect is considerably reduced with coccolith $\delta^{18}O_c$ tending towards inorganic values over the 2 to 12 mmol / kg of DIC range. Not only are interspecies ^{18}O vital effects reduced at high DIC, but also as is the case for carbon isotopes, the absolute vital effects become significantly reduced at the highest DIC level (Fig. 2b). There is, however, a residual +1.3 ‰ $\delta^{18}O$ shift for *E. huxleyi* at 12 mmol / kg of DIC, yet representing a substantial decrease in the magnitude of the vital effect compared to the 2 mmol / kg measurement. The large species *C. pelagicus*, assigned to a near-inorganic (sensu Kim and O'Neil, 1997) group shows constant $\delta^{18}O$ values with a limited vital effect (+ 0.45 ‰), regardless of changes in ambient DIC concentrations.

4 Discussion

4.1 Nature of observed isotopic changes: inorganic or vital effect?

In biological systems, an increase in the DIC concentration of the ambient medium may not be linearly related to that of the mineralising fluid due to the effects of physiology (vital effect). The observation of such contrasting interspecific responses in μ, $\delta^{13}C$ and $\delta^{18}O$ with increased DIC levels in different species points towards a biological control. That the light group increases and the heavy group decreases coccolith $\delta^{13}C$ and $\delta^{18}O$ values precludes a unified thermodynamic mechanism, as the direction of isotopic changes with increased DIC are opposite (Fig. 2a; Fig. 2b). Likewise, we cannot explain the isotopic data of coccoliths by a shift in the relative assimilation of HCO_3^- and CO_2 by the cells with changing ambient DIC concentration (Kottmeier et al., 2014).

Theoretical work and experiments seeking to identify the control of inorganic calcite isotopes have provided useful reference points that are valuable to understand biogeochemical signals and the magnitude of the vital effect. For the carbon isotope system, calcite $\delta^{13}C$ composition is insensitive to temperature, precipitation rates and geologically relevant seawater pH values (Romanek et al., 1992). Thermodynamically, the mechanisms and the dynamics of oxygen isotope fractionation are very different to those for the carbon isotopes (Zeebe and Wolf-Gladrow, 2001). Large isotopic kinetic effects are documented with high precipitation rates favouring ^{16}O incorporation into the calcite crystal (Gabitov et al., 2012). This effect can be as high as 1.5 ‰ for $\delta^{18}O$ values, and corresponds to the "kinetic limit" by Watkins et al. (2013, 2014). An understanding of the saturation state with respect to calcite in the coccolith vesicle, and of true calcite precipitation rates is currently lacking. Both of these concepts are relevant for understanding the vital effect.

The present dataset is not sufficient to tackle whether coccolithophore calcite isotopically derives from a CO_2 or a HCO_3^- source, as it would have required measurement of coeval $\delta^{13}C_{org}$ values. Current literature points towards a mixture of these two DIC species for calcification (e.g. Kottmeier et al., 2014). In the following account, we develop an empirical approach on stable isotopes in coccoliths. Our primary aim here is to better interpret fossil coccolith isotopic signals in the context of DIC availability in the past, without making a hypothesis on which DIC species is used.

4.2 From carbon availability for the cell to the expression of vital effect

4.2.1 Carbon isotope system

In photosynthetic, or photosynthetic-associated biomineralisers such as the foraminifera, corals and coccolithophores, a ^{12}C-DIC depletion of the internal carbon pool due to photosynthetic fractionation by the enzyme Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) may imprint the whole internal carbon pool (*Ci*) leading to substantial isotopic consequences on the stable isotope composition of biominerals (McConnaughey, 1989; Spero et al., 1997; Hermoso et al., 2014).

The species *E. huxleyi* and *P. placolithoides* show particularly high calcite $\delta^{13}C$ values, isotopically higher than both the possible HCO_3^- and $CO_{2\,aq}$ sources (for reference: $\Delta^{13}C_{CO2\,aq\,-\,HCO3^-}$ ~ 9 ‰ and $\Delta^{13}C_{calcite–HCO3^-}$ ~ 1 ‰ at 15 °C; Zeebe and Wolf-Gladrow, 2001). By contrast, *C. pelagicus* and *C. leptoporus* have lower carbon isotopic composition at preindustrial (2 mmol / kg) DIC level, falling between $\delta^{13}C$ of $CO_{2\,aq}$ and $\delta^{13}C$ of HCO_3^-, hence with a lower carbon isotope composition than the inorganic calcite (Fig. 2a).

In species characterised by low PIC/POC, typically *E. huxleyi*, the internal DIC pool is isotopically offset towards high $\delta^{13}C$ values due to intense preferential ^{12}C fixation by photosynthesis (Laws et al., 2002; Benthien et al., 2007; Hermoso et al., 2014; Tchernov et al., 2014). In the culture experiments on *E. huxleyi* by Bach et al. (2013), PIC/POC ratios increased in response to increasing [DIC], which was explained by a decrease in the production of POC. Thus, the lowered *E. huxleyi* $\delta^{13}C$ measured at high DIC concentrations in the present study are the likely consequence of the internal carbon pool becoming less imprinted by ^{12}C photosynthetic-driven Rayleigh fractionation because the latter process is "diluted" in a larger internal carbon pool. We must note, however, that there are substantial discrepancies in the reported $\delta^{13}C_c - \delta^{13}C_{DIC}$ values for *E. huxleyi* between the present study and that by Rost et al. (2002) (Fig. 2a). These differences can be tentatively assigned to divergences in the experimental setup, such as a combined effect of [$CO_{2\,aq}$] and pH in the dataset by Rost et al. (2002), pointing towards a more complex control on the expression of the vital effect than that exerted merely by CO_2 availability.

Species originally with very high $\delta^{13}C$ values at 2 mmol / kg of DIC show a clear increase in their coccolith carbon isotopic ratios with increasing DIC. The increase in *C. leptoporus* and *C. pelagicus* $\delta^{13}C$ with increased DIC is well-correlated with DIC concentrations (Fig. 2a). It is surprising to observe a clear decrease of specific growth rates of *C. leptoporus and C. pelagicus* with more carbon resource in the medium. It has been suggested that intense calcification in *C. pelagicus* may impair growth under high DIC levels due to the challenge to translocate protons outside the cells (Rickaby et al., 2010; Hermoso, 2015). The explanation for higher $\delta^{13}C$ values of *C. leptoporus* and *C. pelagicus* is likely to be common, as with more DIC, a decrease of the PIC/POC ratio is observed in both species (Rickaby et al., 2010; Langer and Bode, 2011; Bach et al., 2013; Diner et al., 2015). With enhanced organic carbon fixation over calcification (*i.e.*, decreased PIC/POC), the whole cell carbon isotopic inventory may become more imprinted by photosynthetic ^{12}C depletion, and as a result, both species produce coccoliths exhibiting isotopically higher carbon isotope signatures, an opposite trend to that observed for *E. huxleyi*.

4.2.2 Oxygen isotope system

It has been hypothesised that the isotopic heavy group is an isotopic relic of a partial CO_2 assimilation by coccolithophore cells (Hermoso et al., 2014). Indeed, CO_2 bears excess ^{18}O atoms compared to DIC and HCO_3^-; the isotopic composition used to compute that of equilibrium calcite (Kim and O'Neil, 1997; Bemis et al. 1998; Zeebe and Wolf-Gladrow, 2001). The fraction of the DIC influx to the cell entering in the form of HCO_3^- does not induce any ^{18}O-enrichment of the *Ci* ($\Delta^{18}O_{CO2\,aq-HCO3^-} = 23.6$ ‰; Zeebe and Wolf-Gladrow, 2001). As the oxygen isotopic composition of inorganic calcite is primarily computed from $\delta^{18}O$ of HCO_3^- (Kim and O'Neil, 1997; Zeebe and Wolf-Gladrow, 2001). Without other thermodynamic effects, a mere acquisition of HCO_3^- by the cell would correspond to equilibrium values. We must add that due to the complexity of the kinetics of the oxygen isotope system, it is, however, impossible to use coccolith $\delta^{18}O$ to quantify the relative supply of DIC by aqueous CO_2 and bicarbonate ions.

Under low ambient DIC level and consecutive carbon limited conditions there may be a fast turnover of the internal carbon pool (Nimer et al., 1992), which allows less time between CO_2 assimilation and calcification in the coccolith vesicle. The residence time of the fraction of the *Ci* built from assimilation of aqueous CO_2 to calcification is fundamental for the extent to which this ^{18}O-rich carbon influx is registered by the coccolith calcite, as it tends to be erased due to isotopic exchange between DIC and H_2O molecules. In a fast growing (calcifying) species, the ^{18}O excess borne by the *Ci* is less isotopically re-equilibrated, and leads to relatively high $\delta^{18}O$ values in coccoliths compared to inorganic calcite or slow growers such as *C. pelagicus*.

In the present study, in all species except *C. pelagicus* that always displays near-inorganic $\delta^{18}O$ values, a link between [DIC] and $\delta^{18}O$ values confirms that the ^{18}O vital effect may be related to the overturning rate (or the "demand-to-supply" ratio, see Bolton and Stoll, 2013). The corresponding isotopic relevant process for the oxygen isotope system is the residence time of the internal carbon pool from cell assimilation of carbon resource to calcification. In *E. huxleyi* with increasing DIC, the record of this ^{18}O excess vanishes, implying that the intracellular residence time of the DIC species in the carbon pool must increase with DIC availability, therefore diminishing the isotopic offset. Comparing our isotope data for *E. huxleyi* and those for *G. oceanica* by Rickaby et al. (2010), we observe that there seems to be an isotopic continuum between the two species based on their isotopic composition / [DIC] relationship (Fig. 3).

For *C. pelagicus*, possible changes of the residence time of the carbon pool prior to its partial mineralisation does not induce expression of an ^{18}O vital effect (Fig. 2b). Near-equilibrium composition of *C. pelagicus* calcite was consistently found under changing temperature and pH conditions (Stevenson et al., 2014; Hermoso, 2015). The expression of very limited ^{18}O vital effect, likely due to the completeness of the oxygen isotope DIC - H_2O exchange at time of calcification in this relatively slow growing species (Hermoso et al., 2014), is a fundamentally important observation with respect to palaeoclimate studies in deep time, due to the geological importance of this near "vital effect-free" species that can be used as a reference.

With this biogeochemical control of oxygen isotope fractionation in coccolith calcite in mind, it remains difficult to explain the lower magnitude of the ^{18}O vital effect for the isotopic "light group" (*C. leptoporus* and *P. placolithoides*). That higher coccolith $\delta^{18}O$ values are recorded with higher ^{18}O-rich CO_2 influx may represent an intuitive reasoning, and reconcile the data. However, their $\delta^{18}O$ values are "capped" by equilibrium values and do not go towards the heavy group end-member as observed in *E. huxleyi* or *G. oceanica* (Fig. 2b), challenging this hypothesis.

4.3 Outlook for coccolith-based palaeoceanographic reconstructions

Using geological evidence in the Neogene, it was reported that large coccoliths exhibit $\delta^{13}C$ values similar to that of planktonic foraminifera whose composition was regarded close to the DIC composition (Bolton et al., 2012). In contrast, small coccoliths were reported to have relatively high $\delta^{13}C$ values in the same study. Our culture data at relatively low DIC concentrations are compatible with these natural environment observations. Furthermore, the present culture-based study confirms the limited expression of ^{13}C vital effect at highest DIC level (Fig. 2a). For coccolith $\delta^{18}O$, the same authors found the opposite: the smallest coccoliths are closest to the foraminifera, and the bigger coccoliths show higher isotopic values. This is also in agreement with the isotopic typology of coccolith calcite, with the notable difference that in culture, larger cells such as *C. pelagicus* exhibit near equilibrium composition. One possible explanation for this discrepancy between culture and sediment data may be the exacerbation of the vital effect in culture due to highly fertilising growth conditions of coccolithophores exposed to high light and nutrient levels (Hermoso et al., 2015). Although the present culture data can be regarded as robust, based on reproducibility of growth and isotope composition in replicated bioassays and thanks to the very dilute cultures undertaken, other carefully conducted studies have reported significantly different carbon isotope fractionation values for *E. huxleyi* using another methodology than that implemented in the present study (Rost et al., 2002; Fig. 2a). This discrepancy highlights that carbon availability cannot be considered as the only driving parameter dictating the magnitude of the vital effects in coccolith calcite. Further experimental work is required to deconvolve the effects of the intricate mechanisms governing them, and ultimately to account for changes in seawater carbonate chemistry as they occur in the "real" oceans. On a similar note, we should stress the importance to consider the whole set of environment parameters, as in our study case, light, nutrient and DIC conditions were likely replete with respect to the natural environment. Under the assumption that in culture, growth rate reached their maxima, it would appear that in the natural environment growth rates were lower, and as a consequence the vital effect, especially for the oxygen isotopes, were also lower.

Using our empirical calibration between the magnitude of the vital effect with DIC concentration or with equivalent pCO_2 (Fig 2a; Fig. 2b; Fig. 3), we validate and encourage the use of coccolith monotaxic to infer SST estimates. The present study indicates that reconstructing meaningful SST estimates from coccolith calcite (and hence, bulk carbonate) $\delta^{18}O$ values requires the a priori knowledge of the range of pCO_2 concentrations for the considered time interval. Further, the data indicate that a constant coefficient of the vital effect cannot homogenously be applied on a coccolith species over its entire geological existence with the notable exception of *Coccolithus pelagicus*. For this species, a unique correction of the ^{18}O vital effect of 0.5 ‰ can be applied on $\delta^{18}O_c$ values to reconstruct SSTs under relatively elevated pCO_2 levels, typically over 600 ppm. Furthermore, it is worth noting that the magnitude of this biological fractionation does not change with pH in this species (Hermoso, 2015). In the dataset of Rickaby et al. (2010), the reported coefficient of the vital effect is the same for *C. pelagicus* at high DIC than in the present study, and for *G. oceanica*, it is of 0.7 ‰ above a 600 ppm threshold.

Exploiting interspecies signals, as the large-small coccolith isotopic offset proposed by Bolton et al. (2012) has the notable advantage to circumvent uncertainties that complicate palaeoceanographic reconstructions (salinity, temperature, seawater $\delta^{18}O$), as they are cancelled out, as they have, at least to first order, a similar effect on coccolith calcite composition. Indeed, considering the arguments presented in this study showing a control by ambient carbon availability and growth dynamics, it appears that the magnitude of the vital effect contains an important environmental parameter sought in palaeoceanography, namely DIC concentrations. Interspecies $\Delta\delta^{18}O$ and $\Delta\delta^{13}C$ offsets with [DIC] can be calculated in the context of the investigated geological period using the data from the present work or those in Rickaby et al. (2010).

The hypothesis by Bolton and Stoll (2013) about a possible "Late Miocene threshold" at about 375 – 575 ppm of atmospheric of CO_2 is expressed in our dataset by a big "jump" in $\delta^{13}C$ value for *Coccolithus pelagicus* (not seen in $\delta^{18}O$ values). In high DIC (elevated atmospheric CO_2) regimes of ocean history with vanished vital effects, departures from the unified +1 ‰ in $\delta^{13}C_c - \delta^{13}C$ values that can be reconstruct with paired coccolith / foraminifera measurements can be used as a proxy for photosynthetic activity in coccolithophores. This approach could complement alkenone-derived palaeo-CO_2 estimates by significantly contribute constraining seawater $\delta^{13}C_{CO2}$ composition and the so-called "*b*" coefficient (Pagani, 2002; Pagani et al., 2005). This novel approach (recently outlined in Hermoso, 2015; Hermoso et al., 2015) will require coupled foraminiferal data that may serve as inorganic reference (Spero et al., 2003). In addition, it appears possible to reconstruct cell geometry via morphometric measurements made on fossil coccoliths (Henderiks and Rickaby, 2007; Henderiks, 2008; Henderiks and Pagani, 2008), as this parameter is of paramount importance for inferring algal growth dynamics and cell size in the absence of preserved coccospheres in the sedimentary register, except in some peculiar settings (Gibbs et al., 2013).

5 Conclusions

This work provides new constraints on the "*mobilis in mobili*" nature of the vital effect in coccolith calcite (Hermoso, 2014). We show that the turnover of carbon and differences in growth rates and potentially relative allocation of the internal pool to photosynthesis and calcification (PIC/POC) concurrently set the magnitude of the vital effect in both carbon and oxygen isotope systems. In coccolithophores, the expression of the vital effect is stronger with a small internal carbon reservoir induced by relatively low ambient carbon concentrations typical of the modern oceans compared to the pCO_2 Neogene history. Several lines of evidence now point towards reduced, if not absent, vital effect under high CO_2 levels, as prevailed during the most of the Meso-Cenozoic. Therefore, the assumption that downcore coccolith $\delta^{18}O$ can be transferred into SST estimates using the equations outlined in Kim and O'Neil (1997) or more recently in Watkins et al. (2013) becomes practical when studying deep time intervals. Due to the complex physiological and environmental control on isotopes in coccolithophores, a fully quantitative modelling approach is now essential, in particular to trace which DIC species are used from the external environment to the coccolith vesicle, and thus refine our understanding of the precise mechanisms behind the vital effect.

Since the pioneering studies on coccolith geochemistry in the 1980s (Anderson and Steinmetz, 1981; Steinmetz and Anderson, 1984; Dudley et al., 1986), a growing body of literature highlights the potential for application to palaeoceanography. Recent work shows major steps towards a complete understanding of the vital effect imprinting isotopes of coccolith calcite based on biogeochemistry and physiology, which may "rival" our quantitative understanding of foraminiferal proxies. These studies and the present work point towards the possibility to generate coccolith-derived long term SST reconstruction and/or pCO_2 levels during periods of abrupt climate change, such as the PETM, Cenozoic climate optima or Mesozoic OAEs.

Acknowledgements

The laboratory work presented in this paper was mostly undertaken by I.Z.X.C., as part of his Master's research project in 2013 under the main supervision and guidance of M.H. We thank Chris Day for help with the isotopic analyses in Oxford and Phil Renforth for seawater alkalinity measurements. We are also grateful to James Rolfe at Cambridge University for his diligence in running carbon isotope analyses of the seawater batches. The authors thank Jelle Bijma for editorial handling, to Lena-Maria Holtz and two other anonymous referees for comments on the discussion paper that have substantially improved the final version of the manuscript. This work was supported by the European Research Council (grant SP2-GA-2008-200915) to R.E.M.R. and by the Natural Environment Research Council (grant NE/H015523/1) to M.H. The article processing charges of this paper have been covered by the OpenAIRE programme.

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Figure captions

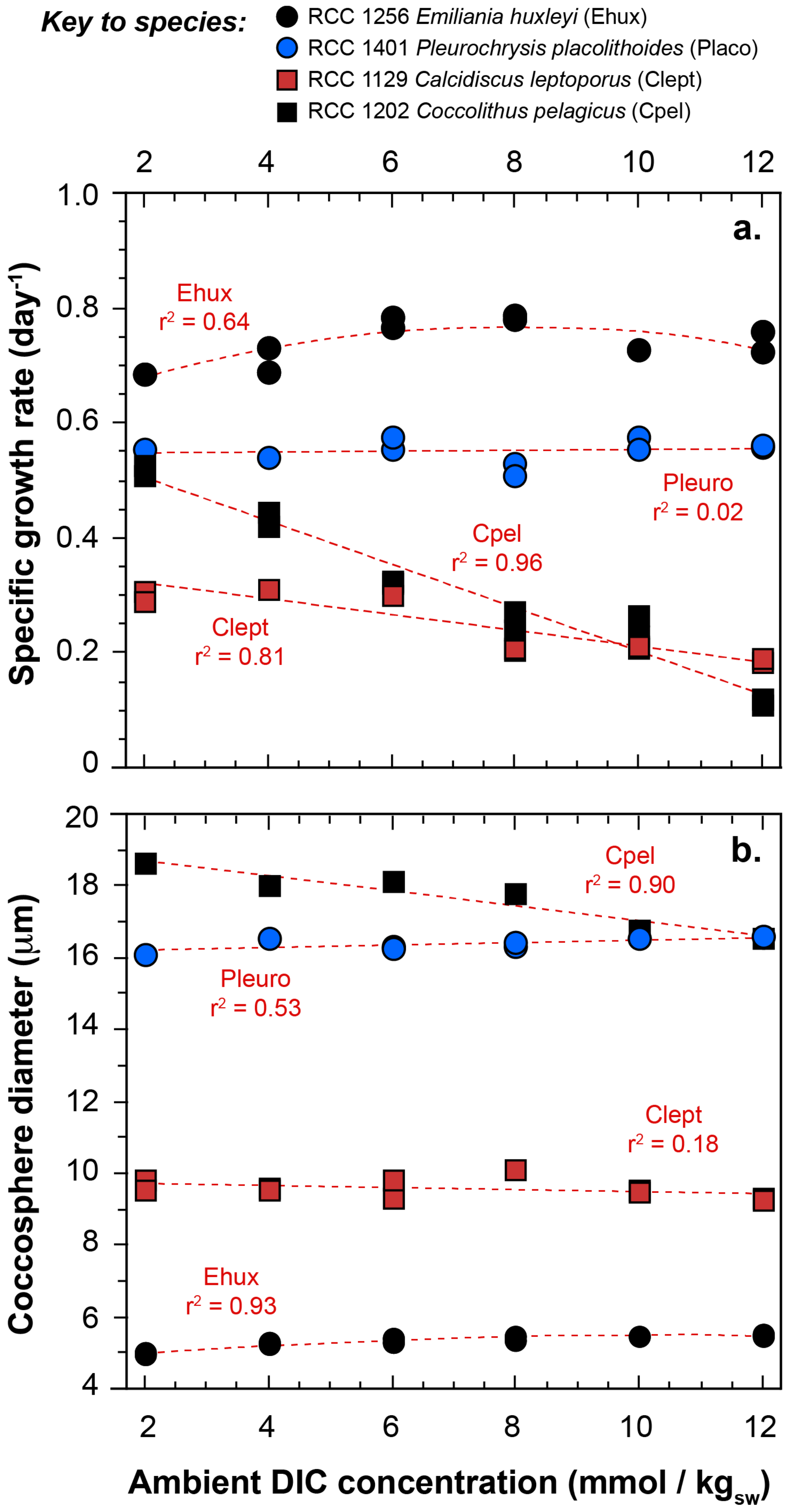
Figure 1. Changes in algae specific growth rates (panel a) and coccosphere diameter (panel b) on a range of 2 to 12 mmol / kg of DIC per kg of seawater in the culture medium. Key for species is inset at the top of the figure.

Figure 2. Changes in coccolith (a) carbon and (b) oxygen isotopes with DIC addition in the culture medium. The results are expressed by isotopic offset of coccolith composition from $\delta^{13}C_{DIC}$ for carbon (panel a) and from medium $\delta^{18}O_{sw}$ for oxygen (panel b). Inorganic calcite references as materialised by the grey horizontal bars on the graphs are calculated according to the equation given by Romanek et al. (1992) and Kim and O'Neil et al. (1997) for carbon and oxygen isotopes, respectively. Correspondence between DIC concentrations and pCO_2 levels were obtained via the CO2Calc software (Table 1). Legend for species is inset. For comparison with previous studies, the grey circles indicate the $\delta^{13}C_c - \delta^{13}C_{DIC}$ values for *E. huxleyi* grown by Rost et al. (2002) – work in which targeted [$CO_{2\ aq}$] were obtained by the titration method (hence pH was left variable between 7.9 and 8.6). Note that only the pCO_2 scale on top of the graph applies for these datapoints.

Figure 3. Scatter plot of carbon and oxygen isotopic offsets with increased DIC concentration. Superimposed on the linear regression lines, the wider side of the red triangles denotes higher DIC level. With increased DIC and aqueous CO_2 concentration in the medium, we observe a clear decrease in the magnitude of isotopic disequilibria in both carbon and oxygen systems, with coccolith isotope compositions converging towards inorganic composition. Note that a correction of +0.64 ‰ was applied to the $\delta^{18}O_c$ values of Rickaby et al. (2010) to account for a temperature offset of +3 °C with the culture data of the present study.

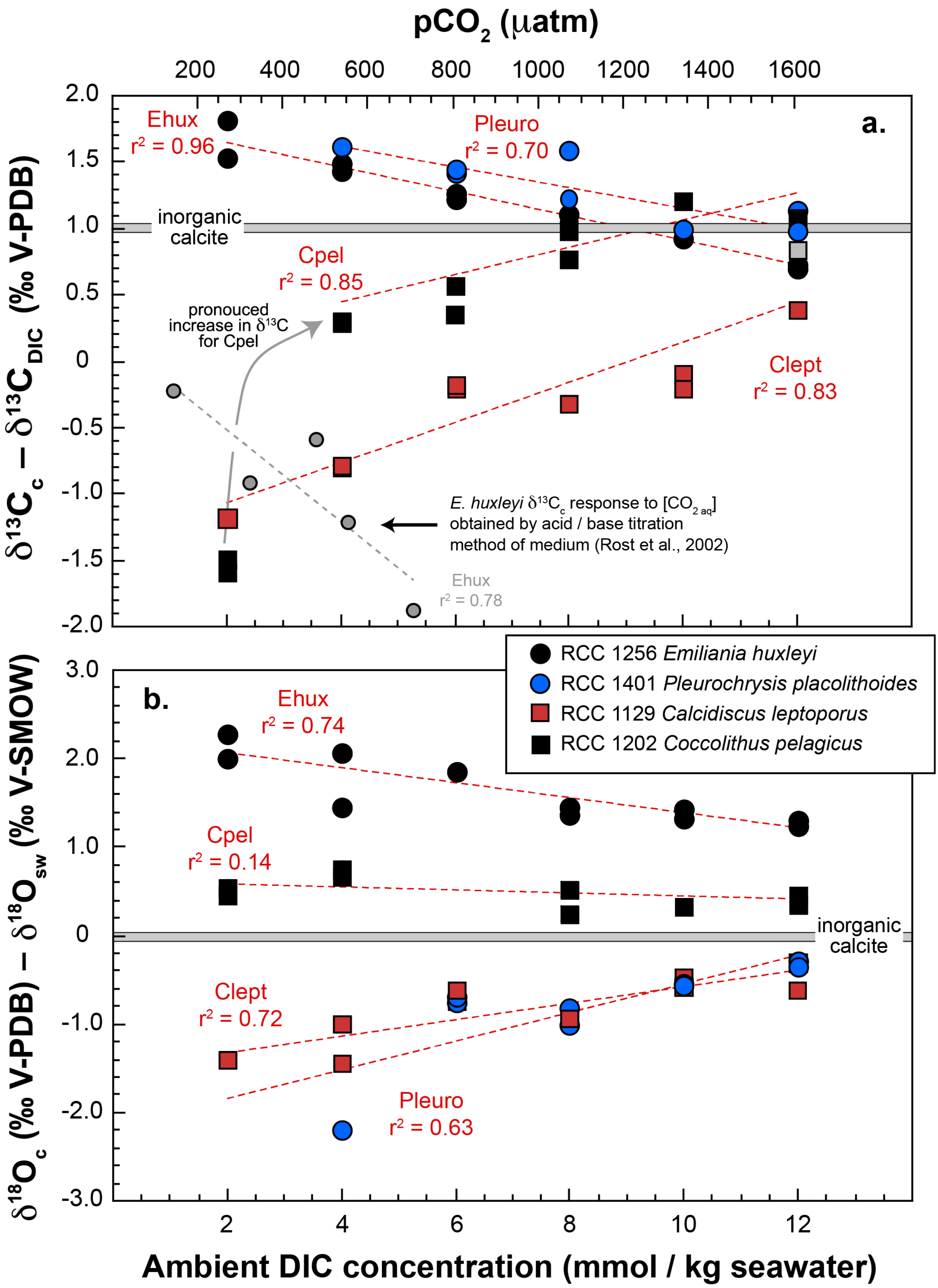
Table 1. Numerical dataset.

Hermoso et al. - Figure 1



1

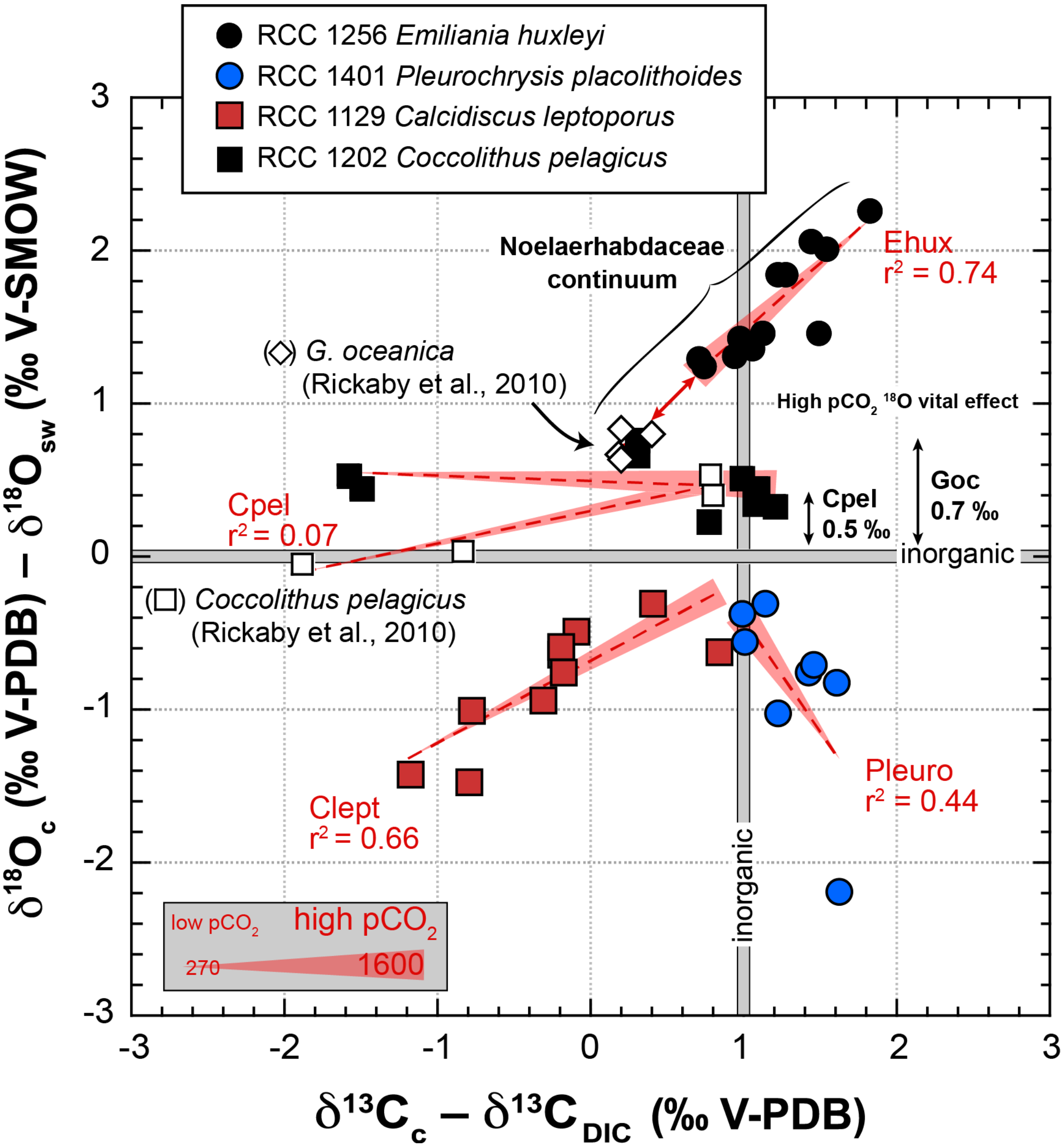
Hermoso et al. - Figure 2



1

2

Hermoso et al. - Figure 3



1

Table 1: Numerical dataset.

	Target DIC mmol kg^{-1}	Temperature °C	Salinity	pH total scale	TA µmol kg^{-1}	$CO_{2\,aq}$ µmol kg^{-1}	HCO_3^- µmol kg^{-1}	CO_3^{2-} µmol kg^{-1}	pCO_2 µatm	$\delta^{18}O_{sw}$ ‰ V-SMOW	$\delta^{13}C_{DIC}$ ‰ V-PDB	$\delta^{13}C_{inorg}$ ‰ V-PDB	$\delta^{18}O_{inorg}$ ‰ V-PDB	$\delta^{13}C_c$ ‰ V-PDB	$\delta^{18}O_c$ ‰ V-PDB	δ-$\delta^{18}O_{c\text{-}sw}$ ‰ V-PDB - V-SMOW	δ-$\delta^{13}C_{c\text{-}DIC}$ ‰ V-PDB	$\varepsilon^{18}{}_{c\text{-}sw}$ *no unit*	$\varepsilon^{13}{}_{c\text{-}DIC}$ *no unit*	μ day^{-1}	Coccosphere diameter µm
Emiliania huxleyi	2	15	33	8.2	2370	10.1	1780.8	209.1	267	0.50	-2.54	-1.54	-0.04	-0.73	2.77	2.27	1.81	29.66	0.81	0.69	4.94
	2	15	33	8.2	2370	10.1	1780.8	209.1	267	0.50	-2.54	-1.54	-0.04	-1.00	2.51	2.01	1.54	29.38	0.54	0.68	5.01
	4	15	33	8.2	4406	20.2	3561.6	418.2	535	0.50	-2.54	-1.54	-0.04	-1.05	1.96	1.46	1.49	29.33	0.49	0.73	5.27
	4	15	33	8.2	4406	19.9	3561.6	418.2	535	0.50	-2.54	-1.54	-0.04	-1.10	2.56	2.06	1.44	29.28	0.44	0.69	5.30
	6	15	33	8.2	6590	30.3	5342.4	627.4	802	0.50	-2.54	-1.54	-0.04	-1.31	2.36	1.86	1.23	29.05	0.23	0.77	5.41
	6	15	33	8.2	6590	29.8	5342.4	627.4	802	0.50	-2.54	-1.54	-0.04	-1.28	2.35	1.85	1.26	29.09	0.26	0.78	5.33
	8	15	33	8.2	8535	40.4	7123.2	836.5	1070	0.50	-2.54	-1.54	-0.04	-1.49	1.87	1.37	1.05	28.87	0.05	0.78	5.37
	8	15	33	8.2	8535	40.4	7123.2	836.5	1070	0.50	-2.54	-1.54	-0.04	-1.43	1.96	1.46	1.11	28.93	0.11	0.79	5.47
	10	15	33	8.2	10853	50.5	8903.9	1045.6	1337	0.50	-2.54	-1.54	-0.04	-1.56	1.94	1.44	0.98	28.80	-0.02	0.73	5.50
	10	15	33	8.2	10853	50.5	8903.9	1045.6	1337	0.50	-2.54	-1.54	-0.04	-1.61	1.82	1.32	0.93	28.75	-0.07	0.73	5.49
	12	15	33	8.2	12963	60.6	10684.7	1254.7	1604	0.50	-2.54	-1.54	-0.04	-1.85	1.81	1.31	0.69	28.51	-0.31	0.76	5.53
	12	15	33	8.2	12963	59.6	10684.7	1254.7	1604	0.50	-2.54	-1.54	-0.04	-1.81	1.75	1.25	0.73	28.55	-0.27	0.72	5.48
Pleurochrysis placolithoides	2	15	33	8.2	2370	10.1	1780.8	209.1	267	0.50	-2.54	-1.54	-0.04	NA	NA					0.56	16.12
	2	15	33	8.2	2370	10.1	1780.8	209.1	267	0.50	-2.54	-1.54	-0.04	NA	NA					NA	NA
	4	15	33	8.2	4406	20.2	3561.6	418.2	535	0.50	-2.54	-1.54	-0.04	NA	NA					NA	NA
	4	15	33	8.2	4406	19.9	3561.6	418.2	535	0.50	-2.54	-1.54	-0.04	-0.92	-1.68	-2.18	1.62	29.47	0.62	0.54	16.55
	6	15	33	8.2	6590	30.3	5342.4	627.4	802	0.50	-2.54	-1.54	-0.04	-1.11	-0.26	-0.76	1.43	29.26	0.43	0.55	16.31
	6	15	33	8.2	6590	29.8	5342.4	627.4	802	0.50	-2.54	-1.54	-0.04	-1.09	-0.19	-0.69	1.45	29.28	0.45	0.58	16.26
	8	15	33	8.2	8535	40.4	7123.2	836.5	1070	0.50	-2.54	-1.54	-0.04	-0.94	-0.32	-0.82	1.60	29.44	0.60	0.53	16.33
	8	15	33	8.2	8535	40.4	7123.2	836.5	1070	0.50	-2.54	-1.54	-0.04	-1.32	-0.51	-1.01	1.22	29.05	0.22	0.51	16.43
	10	15	33	8.2	10853	50.5	8903.9	1045.6	1337	0.50	-2.54	-1.54	-0.04	-1.54	-0.05	-0.55	1.00	28.82	0.00	0.58	16.56
	10	15	33	8.2	10853	50.5	8903.9	1045.6	1337	0.50	-2.54	-1.54	-0.04	-1.54	-0.05	-0.55	1.00	28.82	0.00	0.56	16.56
	12	15	33	8.2	12963	60.6	10684.7	1254.7	1604	0.50	-2.54	-1.54	-0.04	-1.40	0.21	-0.29	1.14	28.96	0.14	0.56	16.53
	12	15	33	8.2	12963	59.6	10684.7	1254.7	1604	0.50	-2.54	-1.54	-0.04	-1.55	0.14	-0.36	0.99	28.81	-0.01	0.56	16.59
Calcidiscus leptoporus	2	15	33	8.2	2370	10.1	1780.8	209.1	267	0.50	-2.54	-1.54	-0.04	NA	NA					0.31	9.83
	2	15	33	8.2	2370	10.1	1780.8	209.1	267	0.50	-2.54	-1.54	-0.04	-3.72	-0.91	-1.41	-1.18	26.57	-2.18	0.29	9.53
	4	15	33	8.2	4406	20.2	3561.6	418.2	535	0.50	-2.54	-1.54	-0.04	-3.34	-0.96	-1.46	-0.80	26.97	-1.80	0.31	9.61
	4	15	33	8.2	4406	19.9	3561.6	418.2	535	0.50	-2.54	-1.54	-0.04	-3.32	-0.51	-1.01	-0.78	26.99	-1.78	0.31	9.54
	6	15	33	8.2	6590	30.3	5342.4	627.4	802	0.50	-2.54	-1.54	-0.04	-2.74	-0.13	-0.63	-0.20	27.58	-1.20	0.30	9.34
	6	15	33	8.2	6590	29.8	5342.4	627.4	802	0.50	-2.54	-1.54	-0.04	-2.72	-0.24	-0.74	-0.18	27.61	-1.18	0.30	9.80
	8	15	33	8.2	8535	40.4	7123.2	836.5	1070	0.50	-2.54	-1.54	-0.04	NA	NA					0.20	10.10
	8	15	33	8.2	8535	40.4	7123.2	836.5	1070	0.50	-2.54	-1.54	-0.04	-2.86	-0.44	-0.94	-0.32	27.46	-1.32	0.21	
	10	15	33	8.2	10853	50.5	8903.9	1045.6	1337	0.50	-2.54	-1.54	-0.04	-2.64	0.02	-0.48	-0.10	27.69	-1.10	0.21	9.53
	10	15	33	8.2	10853	50.5	8903.9	1045.6	1337	0.50	-2.54	-1.54	-0.04	-2.75	-0.09	-0.59	-0.21	27.58	-1.21	0.21	9.48
	12	15	33	8.2	12963	60.6	10684.7	1254.7	1604	0.50	-2.54	-1.54	-0.04	-1.70	-0.11	-0.61	0.84	28.66	-0.16	0.18	9.30
	12	15	33	8.2	12963	59.6	10684.7	1254.7	1604	0.50	-2.54	-1.54	-0.04	-2.15	0.20	-0.30	0.39	28.20	-0.61	0.19	9.24
Coccolithus pelagicus	2	15	33	8.2	2370	10.1	1780.8	209.1	267	0.50	-2.54	-1.54	-0.04	-4.13	1.04	0.54	-1.59	26.15	-2.59	0.53	18.65
	2	15	33	8.2	2370	10.1	1780.8	209.1	267	0.50	-2.54	-1.54	-0.04	-4.04	0.95	0.45	-1.50	26.25	-2.50	0.51	18.65
	4	15	33	8.2	4406	20.2	3561.6	418.2	535	0.50	-2.54	-1.54	-0.04	-2.24	1.26	0.76	0.30	28.10	-0.70	0.45	18.03
	4	15	33	8.2	4406	19.9	3561.6	418.2	535	0.50	-2.54	-1.54	-0.04	-2.25	1.17	0.67	0.29	28.09	-0.71	0.42	18.03
	6	15	33	8.2	6590	30.3	5342.4	627.4	802	0.50	-2.54	-1.54	-0.04	-2.03	NA		0.51			0.33	18.14
	6	15	33	8.2	6590	29.8	5342.4	627.4	802	0.50	-2.54	-1.54	-0.04	-2.15	NA		0.39			0.32	18.14
	8	15	33	8.2	8535	40.4	7123.2	836.5	1070	0.50	-2.54	-1.54	-0.04	-1.55	1.02	0.52	0.99	28.81	-0.01	0.27	17.80
	8	15	33	8.2	8535	40.4	7123.2	836.5	1070	0.50	-2.54	-1.54	-0.04	-1.77	0.74	0.24	0.77	28.59	-0.23	0.24	17.80
	10	15	33	8.2	10853	50.5	8903.9	1045.6	1337	0.50	-2.54	-1.54	-0.04	-1.33	0.84	0.34	1.21	29.03	0.21	0.27	16.76
	10	15	33	8.2	10853	50.5	8903.9	1045.6	1337	0.50	-2.54	-1.54	-0.04	NA	NA					0.23	16.76
	12	15	33	8.2	12963	60.6	10684.7	1254.7	1604	0.50	-2.54	-1.54	-0.04	-1.45	0.96	0.46	1.09	28.91	0.09	0.11	16.58
	12	15	33	8.2	12963	59.6	10684.7	1254.7	1604	0.50	-2.54	-1.54	-0.04	-1.48	0.86	0.36	1.06	28.88	0.06	0.12	16.58