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Coccolith-derived isotopic proxies in palaeoceanography: where geologists need biologists

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Abstract – Coccolithophore biominerals, the coccoliths, represent an important part of the Meso-Cenozoic sedimentary archive. Geochemical analyses of coccoliths can be used to unravel climatic fluctuations in the oceanic realm, but such reconstructions are complicated due to the problem of the "vital effect". This concept refers to the modulation in the record of the physico-chemistry of seawater in calcite due to algal physiology. For decades, it was thought that the magnitude of the vital effect was species-specific and constant for a given species. Recent studies aiming at a mechanistic understanding of these processes point towards a plastic and environmental-dependent interplay between the physiology of coccolithophores and isotopic composition in coccolith calcite. This "*mobilis in mobili*" relationship opens the door to the possibility to explore the vital effects as palaeoenvironmental proxies undertaking an interspecies approach. New physiological parameters, such as the quantification of calcification rates, pH, and calcium and carbon pools in the coccolith vesicle would further help geologists to constrain the vital effect. Emerging "non-traditional" isotope systems will also contribute to refine the transfer functions between coccolith geochemistry, vital effect, and palaeoenvironments.

Coccolith / biomineralisation / CO_2 concentrating mechanism / vital effect / culture / pelagic sediment / isotope fractionation / climate proxies

Résumé – Les biominéraux produits par les coccolithophoridés, ou coccolithes, représentent une part importante de l'archive sédimentaire du Méso-Cénozoïque. Les analyses géochimiques de coccolithes peuvent être utilisées pour reconstruire les fluctuations climatiques du milieu océanique, mais ces mesures doivent être déconvoluées du problème de l'« effet vital ». Ce concept fait référence à la modulation de l'enregistrement de la physico-chimie de l'eau de mer dans la calcite par la physiologie des algues. Pendant des décennies, il a été pensé que l'intensité de l'effet vital était spécifique à l'espèce et constante pour une espèce donné. Des travaux récents visant à la compréhension des mécanismes de ces processus montrent une plasticité qui dépend de l'effet de l'environnement sur la physiologie des coccolithophoridés et à terme, sur la composition isotopique des coccolithes. Cette relation « *mobilis in mobili* » ouvre la voie de l'exploration des effets vitaux comme marqueurs paléoenvironnementaux en entreprenant une démarche interspécifique De nouvelles contraintes sur la physiologie de ces algues comme la quantification des vitesses de calcification, du pH, de la taille des réservoirs de calcium et de carbone dans la vésicule de calcification aideraient les géologues à mieux contraindre l'effet vital. Des systèmes isotopiques dits « non-traditionnels » en plein essor contribueront également à affiner les fonctions de transfert entre la géochimie des coccolithes, les effets vitaux, et les paléoenvironnements.

Coccolithe / biominéralisation / méchanisme de concentration de CO_2 / effet vital / culture / sédiment pélagique / fractionnement isotopique / traceurs environnementaux

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INTRODUCTION

Calcification in coccolithophores has provided a valuable sedimentary archive since the appearance of this clade of the Haptophyta in the fossil record dated to the Late Triassic (Bown & Cooper, 1998). Large-scale accumulation of coccoliths, as evidenced by the deposition of chalk formations during the Cretaceous, illustrates the rock-forming role of these biominerals. Geologists from both the proxy and modelling sectors have interest in calcite micro- and nannofossils (foraminifera, coccoliths and calcareous dinoflagellates cysts) as they represent most of the sedimentary material in the open-ocean realm. Coccolith-based palaeoenvironmental reconstruction has mainly been achieved by studying assemblages in pelagic sediments. The geochemistry of coccoliths (isotopic composition and trace metal analyses) has been largely overlooked due to methodological and conceptual issues. Coccoliths have not been the subject of the boom in palaeoceanographic studies witnessed for the foraminifera for two main reasons: *i*) their minute size that makes their extraction from sediments very challenging, and *ii*) the problem of the vital effect.

Recent techniques for concentrating near-specific calcareous nannofossil assemblages from sediments have transcended technical difficulties due to the micron-sized nature of coccoliths (Minoletti *et al*., 2001; Stoll & Ziveri, 2002). These methodologies enable generation of diagenetically-screened isotopic signals, reflecting those of surface ocean layers (Minoletti *et al*., 2005; Stoll, 2005; Beltran *et al*., 2007; Hermoso *et al*., 2009a, 2009b; Turpin *et al*., 2011; Bolton *et al*., 2012; Rousselle *et al*., 2013). The next step that would allow a full understanding of the signal born by these coccolith fractions is further investigation of the vital effect. In particular, it is essential to determinate to what extent geochemical measurements made on coccolith-enriched or mono-specific assemblages reflect the physico-chemical composition of seawater in the present and in the past. The suite of physiologically-mediated reactions in the cell from uptake to intracellular calcification remains largely unconstrained. Palaeoenvironmental interpretations from nannofossil geochemistry are hence somewhat elusive, explaining why coccoliths have been overlooked for palaeoceanographic studies over the last six decades.

The study of coccoliths embraces a wide range of disciplines spanning from characterisation of their formation (physiology and biomineralisation) to their deposition and preservation in pelagic sediments (sedimentology). Unlike foraminifera, the coccolithophores are relatively easily to culture in the laboratory (Probert & Houdan, 2004). Interest in their response to ongoing ocean acidification (Riebesell *et al*., 2000; Iglesias-Rodriguez *et al*., 2008; Bach *et al*., 2013) has promoted research on the physiology of these organisms. It is also possible to attempt culture calibrations of isotope fractionation and trace metal partitioning in coccoliths in response to different parameters, such as the relationship between oxygen isotopes ($\delta^{18}O$) and temperature. Palaeoenvironmental reconstructions based on coccolith geochemistry rely on transfer functions between isotopic or elemental measurements of coccoliths and primary (seawater) signals. To fully exploit these empirical calibrations, and to be in a position to transfer them to the fossil record, we still need to gain a full understanding of the vital effect that is prone to substantially imprinting the composition of the coccoliths (Dudley *et al*., 1986; Stoll & Ziveri, 2004).

In the modern ocean, marine algae using C3 photosynthetic pathways face carbon (CO_2) limitation, as is the case for a number of coccolithophore

species. Active mechanisms to increase intracellular CO_2 concentrations have evolved (known as "carbon concentrating mechanisms" or CCMs; Giordano *et al.*, 2005), and are thought to play a role in the expression of vital effects in coccolith calcite (Bolton & Stoll, 2013).

This paper presents an overview of our current understanding of the vital effect in coccoliths with a geological perspective, mainly focusing on carbon and oxygen isotopes, and highlights current limitations in the use of coccolith-based proxies that could be transcended by further constraining physiological processes involved in intracellular calcification in coccolithophores.

THE ISOTOPIC JARGON IN CALCITE ISOTOPE GEOCHEMISTRY

The use of isotopic proxies relies on the comparison of isotopic composition of the mineral (calcite in the case of the coccoliths) and that of the mineralising fluid, or more pragmatically that of seawater or culture medium. The isotopic composition of calcite is the reflexion of the relative abundance of two isotopes, usually the light (abundant) over the heavy (rare), as is the case for the $^{12}C/^{13}C$ and $^{16}O/^{18}O$ isotopic systems. Isotopic compositions are expressed relative to an international standard. The "δ" notation – followed by the heaviest isotopes and the element, e.g. $\delta^{18}O$ – is obtained by the formula exemplified on the oxygen isotope system (Eq. 1).

$$\delta^{18}O_{coccolith}\,[‰] = ([\delta^{18}O_{coccolith} - \delta^{18}O_{standard}] / \delta^{18}O_{standard}) \times 1000 \qquad \text{(Eq. 1)}$$

In order to assess how isotopes are distributed (fractionated) from seawater into calcite of the coccoliths, the fractionation coefficient (termed "α") is the ratio between the compositions (relative abundances) of the mineral over that of the fluid (expressed in the same unit and against the same standard) (Eq. 2; Eq. 3).

$$^{18}\alpha_{coccolith\text{-}seawater}\,[\text{no unit}] = (^{16}O/^{18}O_{coccolith}) / (^{16}O/^{18}O_{seawater}) \qquad \text{(Eq. 2)}$$

or, by approximation via the "δ" notation:

$$^{18}\alpha_{coccolith\text{-}seawater} = \delta^{18}O_{coccolith} / \delta^{18}O_{seawater} \qquad \text{(Eq. 3)}$$

The fractionation coefficient, termed "ε", can be calculated for α close to 1 as (Eq. 4):

$$\varepsilon_{coccolith\text{-}seawater}\,[‰] = (\alpha - 1) \times 1000 \qquad \text{(Eq. 4)}$$

Informally – and to some extent improperly – the magnitude of fractionation can be expressed as the difference of the isotopic composition of calcite and seawater, in which case "δ" does not necessarily have to be expressed in the same scale. The "δ – δ" notation is commonly used in palaeoceanographic studies, for example as a palaeotemperature proxy using oxygen isotope composition of calcite (e.g. Bemis *et al.*, 1998).

There are two types of physiologically-induced fractionations. The first is the thermodynamic equilibrium of a given substance that will eventually be incorporated into the calcite lattice. If we compare carbon isotopes of CO_2 and HCO_3^- molecules, $\delta^{13}C$ values differ in both cases because carbon atoms are bound differently to surrounding atoms. This indicates that the dissolved inorganic carbon (DIC) assimilated by a cell may influence $\delta^{13}C$ of coccolith calcite. The second phenomenon is kinetic fractionation and applies when atoms diffuse or are transported through a transmembrane channel pump for example, as a ^{12}C-^{16}O-^{18}O CO_2 molecule will move faster than a ^{13}C-^{18}O-^{18}O CO_2 molecule. Kinetic effects induce the light isotopes to be incorporated predominantly into the next cytosolic compartment or eventually into calcite.

The concept of the vital effect comprises all of the physiological phenomena that influence the isotopic composition of the coccolith. As such, the vital effect is an abstract sum of mass dependent fractionation (equilibrium and kinetic) steps between uptake and mineralisation of cations (mainly Ca^{2+}, Mg^{2+} and Sr^{2+}) and DIC species from which coccoliths are built. The vital effect is not the offset between coccolith and extracellular (seawater) elemental and isotopic composition for a given substance, as this concept should not include thermodynamic (abiogenic) effects that impact calcification. Hence, the vital effect can be quantified by the difference between the isotopic composition of coccoliths and that of an inorganically-precipitated calcite (Eq. 5; Eq. 6).

$$^{18}O \text{ vital effect } [‰] = (\delta^{18}O_{coccolith} - \delta^{18}O_{seawater}) - (\delta^{18}O_{equilibrium} - \delta^{18}O_{seawater}) \quad \text{(Eq. 5)}$$

$$= \delta^{18}O_{coccolith} - \delta^{18}O_{equilibrium} \quad \text{(Eq. 6)}$$

The vital effect also represents a multi-component signal, including various fractionation steps that can have opposite directions, and whose individual effects may cancel each other. We can only assess a bulk effect by measuring initial (liquid) and final (solid) geochemical signatures. Measurement of an isotopic composition in a coccolith close to equilibrium conditions does not necessarily mean that there is no vital effect at play. This illustrates the conceptual complexity of the understanding of the vital effect with strong specificities for each isotopic system.

Another complication arises from the difference between fractionation and apparent fractionation. If the vital effect is understood as physiologically-mediated fractionation, it has to be regarded as a black box, as we can only measure the difference between the extracellular environment and the coccolith, with large uncertainties concerning the composition of the mineralising fluid. However, the physico-chemistry of the mineralising fluid, which is under strong biological control, will greatly influence the subsequent fractionation that will occur during calcification. This artefact is well illustrated by the carbon isotope system, which is characterised by a strong interplay between calcification and photosynthesis (Hinga *et al*., 1994; Benthien *et al*., 2007; Bolton & Stoll, 2013; Hermoso *et al*., 2014; Fig. 1).

In downcore studies, differential (interspecific) vital effects assume that all coccolith species from an assemblage have an identical extracellular environment in terms of chemical composition, pH and temperature, and virtually the same inorganic reference. This concept is supposed to only reflect a modulation merely due to physiological processes and can be used as such when exploring the nannofossil record.

TECHNIQUES FOR STUDYING COCCOLITH GEOCHEMISTRY

Laboratory cultures

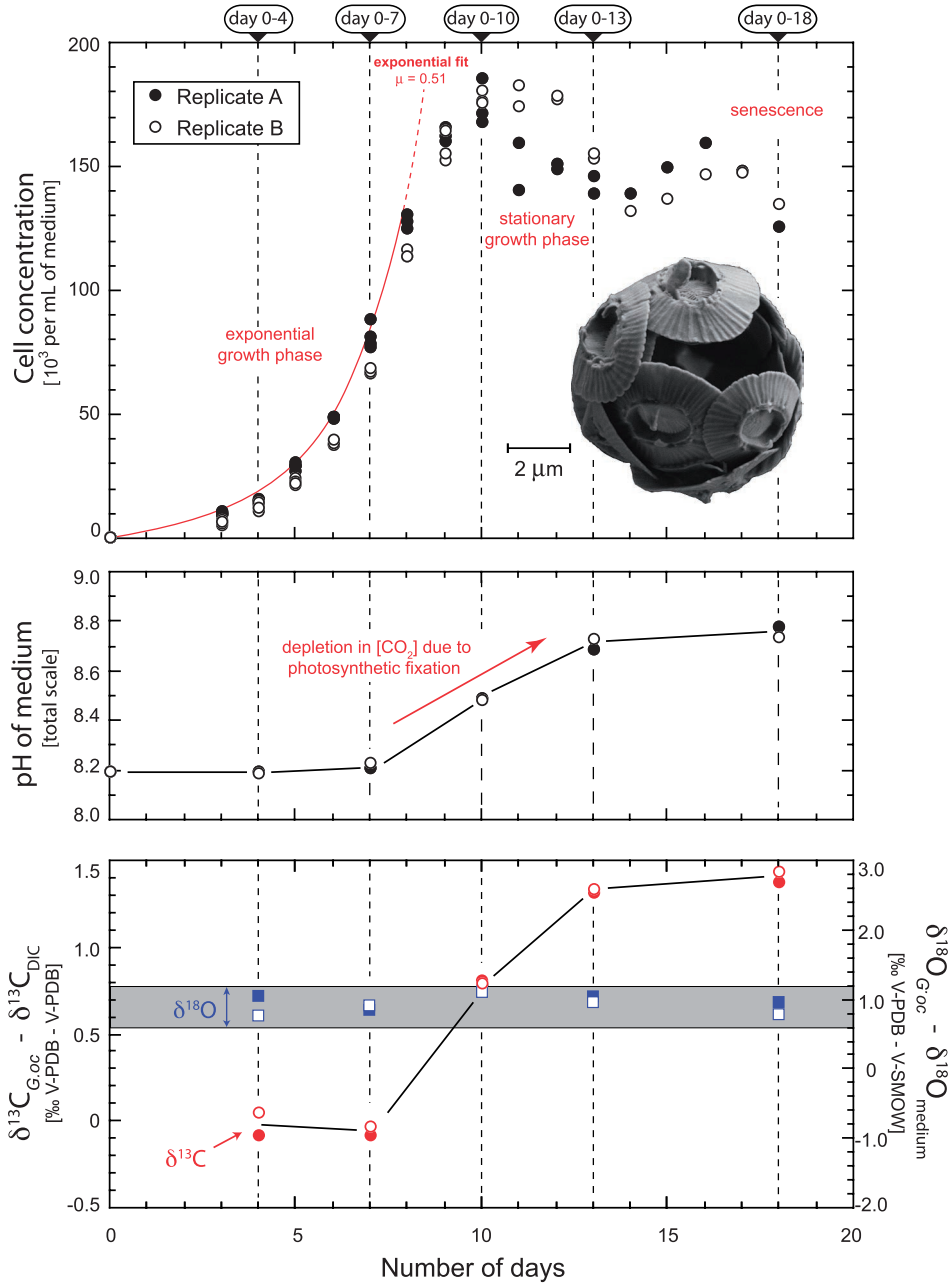
A wide range of coccolithophore strains is available at various culture repositories, notably the Roscoff Culture Collection (http://www.roscoff-culture-collection.org), enabling intra- and inter-specific comparisons. In the laboratory, it is relatively easy to manipulate the environmental conditions that algal cells are exposed to, and subsequently measure how these affect isotopes. To date, the parameters tested "only" include temperature, DIC concentration, light level and $\delta^{18}O$ of the culture medium (Dudley & Goodney, 1979; Dudley *et al*., 1986; Ziveri *et al*., 2003; Rickaby *et al*., 2010; Hermoso *et al*., 2014 and references therein).

The majority of culture studies with a geochemical perspective are undertaken in batch culture conditions, which are easy to implement (Fig. 1). There is, however, a number a biases spanning from purely inorganic to physiological effects that can change the isotopic composition of the medium and of coccoliths if batch cultures are not harvested at very dilute cell concentration (Hinga *et al*., 1994; Riebesell *et al*., 2008; Barry *et al*., 2010; Hermoso *et al*., 2013, 2014; Fig. 1).

The most studied coccolithophore model organism in geochemical, physiological and molecular contexts is *Emiliania huxleyi*. This is due to the dominance of this species in modern oceans, and also, to some extent, to the fact that it grows rapidly in culture. As discussed below, regarding a particular monoclonal strain of *E. huxleyi* as representative of this species or even of an integrated coccolith community is questionnable. Many intraspecific physiological differences exist for this taxon (Langer *et al*., 2006). Furthermore, *E. huxleyi* recently evolved in the fossil record and became dominant only about 70 000 years ago. This species is presumably adapted for present-day conditions in the water column (e.g., low CO_2 concentration), meaning that extrapolating findings made on this species to the Cenozoic does not appear to be an acceptable assumption with a geological perspective.

Coccolith oozes

Coccoliths have not known the same development as foraminifera in terms of palaeoceanographic use due to their minute size (2 to 20 μm) and the difficulty to isolate them easily from sediments. However, owing to their dominance in the calcite fraction of pelagic sediments, geochemical analyses performed on the whole carbonate fraction more or less reflect the signature of mixed coccolith assemblages. Coccoliths can be concentrated from sediment by wet micro-sieving. The coarse fraction consisting of foraminifera can be discarded or separately analysed, whereas the fine fraction, usually less than 63 μm or 20 μm, predominantly comprises coccoliths (Anderson & Steinmetz, 1981; Paull & Thierstein, 1987; Ennuy *et al*., 2002; Chiu & Broecker, 2008; Minoletti *et al*., 2009). In addition to coccoliths, calcareous dinoflagellate shells and foraminiferal fragments, the calcite part of the fine fraction also contains the so-called micarbs (Bellanca *et al*., 1997; Mattioli & Pittet; 2002; Beltran *et al*., 2009; Minoletti *et al*., 2009). These abundant infra micron-sized particles may have various origins, and be isotopically very different from coccoliths. They have been reported to have relatively high oxygen isotope ratios that would potentially indicate a cold water signature (Minoletti *et al*., 2009).


day 0-4
day 0-7
day 0-10
day 0-13
day 0-18
Replicate A
Replicate B
exponential fit
μ = 0.51
senescence
stationary growth phase
exponential growth phase
2 μm
Cell concentration
[10³ per mL of medium]
depletion in [CO₂] due to photosynthetic fixation
pH of medium
[total scale]
δ¹⁸O
δ¹³C
δ¹³C G.oc - δ¹³C DIC
[‰ V-PDB - V-PDB]
δ¹⁸O G.oc - δ¹⁸O medium
[‰ V-PDB - V-SMOW]
Number of days

Coccolith microfractions

Two main protocols have been developed with a view to concentrating near monospecific assemblages of coccoliths from highly heterogeneous sediment samples: that of Minoletti *et al*. (2001, 2009) based on cascade microfiltering steps (Fig. 2), and that of Stoll & Ziveri (2002) based on a decanting technique. Subsequently, more sophisticated protocols have been designed for trace metal analyses in coccoliths (Stoll *et al*., 2007; Halloran *et al*., 2009). The utilisation of these two latter sorting protocols using micropicking and flow cytometry, respectively, remains rather scare, probably due to their intricate implementation, and the fact they only allow measurements of microquantities of calcite.

Core top calibration represents a necessary intermediate step between culture and downcore studies to ensure that findings are transferable to the geological record. Such an approach requires making assumptions on the ecology of coccolithophores (seasonality and calcification depth), on associated physico-chemical parameters (temperature, $\delta^{18}O_{sw}$, $\delta^{13}C_{DIC}$, alkalinity, pH) and on the purity of microseparated coccolith assemblages. It is also necessary to ensure that coccoliths are sufficiently recent to compare their isotopes to present-day conditions in the water column. This can be achieved via ^{14}C dating or assessment of the sedimentation rate that needs to be as high as possible. For temperature and $\delta^{18}O_{sw}$, a maximum age of 6,000 years seems to be reasonable limit beyond which calcite may have recorded the last deglaciation. Correcting carbon isotopes is trickier due to the anthropogenic ^{12}C-rich CO_2 invasion of the surface of the ocean that is occurring since the industrial revolution, the so-called "Suess effect" (Baxter & Walton, 1970). Some of the coccoliths present in core top samples were produced before this phenomenon started influencing seawater chemistry, hence in waters with $\delta^{13}C_{DIC}$ higher than present-day compositions available in oceanic databases (e.g., Tagliabue & Bopp, 2008). Overall, the global effect of this phenomenon is comprised in a range between + 0.5 to + 2.5‰ depending on regional settings (Gruber *et al*., 2009). Using foraminiferal isotopic evidence, a ~ + 2‰ offset in temperate and non-upwelling regions was calculated by Spero *et al*. (2003).

Lastly, core top studies enable examination of a population of coccoliths rather than of a monoclonal strain of a given species. Hence, examining the natural environment enables determination of whether or not clones are representative of a whole taxonomic level.

◀ Fig. 1. Evolution of *Gephyrocapsa oceanica* (strain RCC 1314) cell density (top panel), pH of medium (medium panel) and measured carbon and oxygen isotope compositions (lower panel) during a continuous batch culture. Five culture flasks (with two replicates for each) were grown in exactly the same conditions (15°C; irradiance ~ 150 µmol photons m^{-2} s^{-1}; in ESAW artificial medium with nutrient K/2 level – see composition in Harrison *et al.* (1980)) and were sequentially harvested at days 4; 7; 10; 13 and 18. The mean average growth rate for this experiment was 0.51 day^{-1} and, cultures were not at their optimum division rate from day 7, probably due to aqueous CO_2 limitation in the medium. The increase in pH can be the consequence of preferential utilisation of aqueous CO_2 and HCO_3^- with respect to CO_3^{2-}. Although there is no effect on oxygen isotope of coccolith calcite with cell concentrations ($\delta^{18}O_{coccolith}$ – $\delta^{18}O_{seawater}$ ~ + 1%), significant alteration of the carbon isotope system was observed with $\delta^{13}C_{coccolith}$ becoming significantly heavy indicating a reservoir effect. Note that each step is incremental and hence depends on previous culture stages. Figure from Hermoso *et al*. (2013).

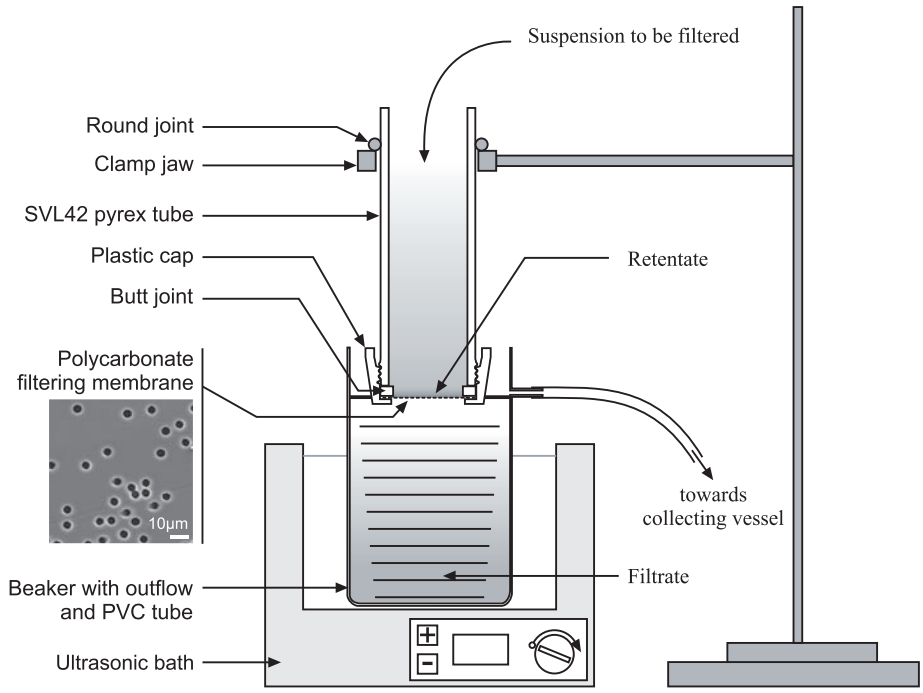


Fig. 2. Microseparation device, the so-called "Minoletti technique", based on cascade microfiltering steps of sediment fine fractions. Screen membranes are made of polycarbonate with well-calibrated nominal apertures (SEM image inset), and the filtering process is facilitated by the use of gentle ultrasonic treatment to avoid membrane clogging. Figure from Minoletti *et al.* (2009). See also Fig. 3 in Bolton *et al.* (2012) for a protocol based on decanting steps.

OVERVIEW OF THE ISOTOPIC SYSTEMS

The two first comprehensive culture studies by Dudley *et al.* (1986) and Ziveri *et al.* (2003) have generated ^{18}O and ^{13}C fractionation coefficients highlighting strong species-specific differences (up to 5‰ in both systems). More recent studies have furthered our understanding of traditional isotopes in coccolith calcite (Rickaby *et al.*, 2010; Ziveri *et al.*, 2012; Candelier *et al.*, 2013; Bolton & Stoll, 2013; Hermoso *et al.*, 2014). With the emergence of new (non traditional) isotopic proxies, cultured coccoliths have been measured for their carbonate clumped isotope composition (Δ_{47}) and calcium ($\delta^{44}Ca$), magnesium ($\delta^{26}Mg$) and stable strontium ($\delta^{88}Sr$) isotopes at different temperatures (Gussone *et al.*, 2006, 2007; Langer *et al.*, 2007; Ra *et al.*, 2010; Tripati *et al.*, 2010; Müller *et al.*, 2011; Stevenson *et al.*, 2014).

Carbon isotope system

Overview

Over long timescales, the use of $\delta^{13}C$ in palaeoceanography is thought to reflect palaeoproductivity via the intensity of organic carbon production and

deposition to the seafloor (Broecker, 1982). The global isotopic carbon cycle is driven by the large $^{13}/^{12}C$ fractionation that occurs during photosynthesis – the organic matter being substantially ^{12}C-enriched (Laws *et al*., 2002).

Coccolithophores combine both calcification and photosynthetic carbon fixation, and it is interesting to note that a similar photosynthetic-driven process is at play at the cellular level (Bolton & Stoll, 2013; Hermoso *et al*., 2014).

Measured carbon isotope fractionation in coccolith calcite is only apparent

The inorganic reference shows little fractionation between the dominant DIC species at pH 8.2 (HCO_3^-) and calcite with an isotopic offset of + 1‰ in the solid phase (Romanek *et al*., 1992) (Eq. 7).

$$\delta^{13}C_{\text{equilibrium calcite}} = \delta^{13}C_{DIC} + 1 \text{ (with } \delta^{13}C_{DIC} \approx \delta^{13}C_{HCO3\text{-}} \text{ at seawater pH} \sim 8) \quad \text{(Eq. 7)}$$

As most marine algae rely on diffusion of CO_2 (either passively or via inducible expression of CCMs such as excretion of carbonic anhydrase), one may expect $\delta^{13}C_{coccolith}$ values to be very negative compared to equilibrium conditions. Indeed, $\delta^{13}C$ of CO_2 is about 10‰ more negative than that of HCO_3^-. However, the $\delta^{13}C$ of *E. huxleyi* is very positive, even more positive than equilibrium. Such high values (Fig. 3) cannot be explained thermodynamically, indicating that the expression of a vital effect affects the carbon isotope system. Due to the large fractionation by RubisCO, photosynthetic carbon fixation favours incorporation of ^{12}C into organic matter, leading to increased ^{13}C concentrations in the intracellular compartments following a progressive Raleigh distillation process. Hence, calcite is ultimately formed from a ^{13}C-rich carbon pool, and $\delta^{13}C$ is anomalously high. By anomalous, it is meant that actual $\delta^{13}C$ is different from that of the culture medium (Fig. 1).

It is worth noting that *Coccolithus pelagicus* shows much more negative $\delta^{13}C$ values than *E. huxleyi* (Ziveri *et al*., 2003; Hermoso *et al*., 2014; Fig. 3). This could be explained by a modulation of this photosynthetic effect with the intensity of photosynthetic carbon fixation compared to calcification. *E. huxleyi* has a much lower Particulate Inorganic Carbon to Particulate Organic Carbon (PIC/POC) ratio, and hence a large positive drift in the internal pool (Hermoso *et al*., 2014). More broadly, it is apparent that a relationship exists between species-specific PIC/POC ratio and the direction of apparent carbon isotope fractionation in coccoliths (Fig. 3).

Estimates of reservoir effect-free $\delta^{13}C_c$ and $^{13}C/^{12}C$ fractionation factors will be particularly challenging. This would only be possible by applying mass balance equations that require extensive knowledge of other parameters, such as the PIC/POC ratio and $\delta^{13}C$ of the organic matter. Such data has not yet been published, but would enable valuable refinement of the carbon isotope systematics in coccolithophores, as initiated by Bolton & Stoll (2013) and Holtz *et al*. (2014).

A large reservoir effect overprints $\delta^{13}C$ values of cultured coccoliths

During continuous cultures, an elevation in $\delta^{13}C$ of the DIC in the medium accompanies growth (Hinga *et al*., 1994; Benthien *et al*., 2007; Moolna & Rickaby, 2012). The $\delta^{13}C$ of coccoliths of *G. oceanica* produced during an incremental continuous culture substantially increases, by + 1.5‰ (Fig. 1).

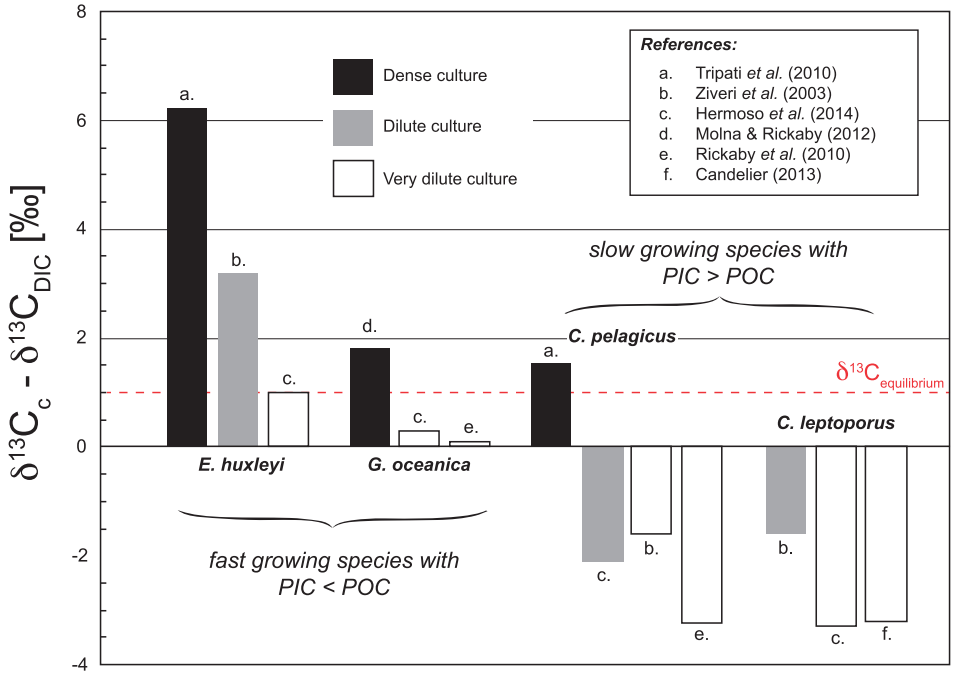


Fig. 3. Compilation of existing carbon isotope compositions of cultured coccoliths. The choice of the culture technique has a great influence on measured $\delta^{13}C_{coccolith}$ (see fig. 1). Relatively small and fast growing coccolithophores such as *Emiliania huxleyi* and *Gephyrocapsa oceanica* produce coccoliths with high $\delta^{13}C$ values owing to a photosynthetic-driven Rayleigh distillation process (preferential incorporation of ^{12}C into the organic matter). By contrast, *Coccolithus pelagicus* (disregarding the dense culture) and *Calcidiscus leptoporus* have $\delta^{13}C_{coccolith}$ closer to the carbon substrate taken up by the cell (aqueous CO_2 signature; $\delta^{13}C \sim -9‰$) because the distillation effect is less operative in these species producing less organic matter. PIC stands for Particular Inorganic Carbon (calcite) and POC for Particular Organic Carbon (organic matter). Carbon isotope composition of equilibrium calcite is calculated after the equation of Romanek *et al.* (1992), see Eq. 7 in text. References are inset upright.

This calls into question the validity of previously published $\varepsilon^{13}C_{cocco\text{-}DIC}$ factors from relatively high cell density batch cultures (Fig. 3).

Figure 1 suggests that there is no or little apparent fractionation in the carbon isotope system assuming a HCO_3^- substrate for calcification for *G. oceanica* (Rickaby *et al.* 2010). Similar values are found during the first half of the exponential phase before the reservoir effect affects both the DIC internal pool and the culture medium (Figs 1 & 3). Overall, this shows that particular attention must be paid when culturing coccolithophores for isotope studies of a small size reservoir such as that of the carbon system. Another phenomenon that can exacerbate this culture artefact comes from the fact that cells are not permanently resuspended in the culture medium. Cells tend to settle at the bottom of the flasks and stick together, leading to substantial boundary layer effects (the alteration of the medium composition immediately around the cell). Even the implementation of semi-continuous batch or even chemostat systems would not fully overcome this caveat.

It is worth noting that this reservoir effect may also bias the determination of RubisCO fractionation in culture (Laws *et al.*, 2002; Boller *et al.*, 2011), as similar positive drift affects both calcification and photosynthesis pathways, and also attempts to quantify carbon isotope fractionation in alkenones, as pointed out by Benthien *et al.* (2007). Alkenones are membrane lipids produced by species within the Noelaerhabdaceae family. Their compound specific isotopic measurements ($\delta^{13}C_{alkenone}$) allow deriving $\varepsilon^{13}C_{alkenone\text{-}CO2}$ or "εp" coefficients that have been found to be a useful palaeo-CO_2 proxy (Pagani, 2002).

Refining intracellular $\delta^{13}C$ and deciphering true fractionation coefficients would greatly improve our knowledge of instantaneous ("non-linear") calcification rates, and alkenone palaeo-CO_2 barometry, as downcore alkenone εp values may be flawed (Zhang *et al.*, 2013).

Influence of the temperature on $\delta^{13}C$ of coccoliths

In inorganic systems, there is no influence of temperature on the carbon fractionation factor ($\varepsilon_{CaCO3\text{-}HCO3\text{-}}$ is near constant), and $\delta^{13}C$ of calcite is offset by + 1‰ with respect to $\delta^{13}C$ of HCO_3^- at a given pH (Romanek *et al.*, 1992). However, there is a slight temperature effect on $\delta^{13}C$ of HCO_3^- with temperature (Mook *et al.*, 1974). In corals and in foraminifera, which mainly assimilate DIC in the form of HCO_3^- by vacuolisation of seawater, this thermodynamic temperature dependence on the isotopic composition of the DIC pool may be registered into calcite. The situation is different in CO_2-utilising calcifiers, such as unicellular marine algae, because carbon isotope composition of aqueous CO_2 relative to atmospheric CO_2 does not change with temperature (Mook *et al.*, 1974). This offset between aqueous CO_2 and HCO_3^- or total DIC is as follows (Eq. 8).

$$\delta^{13}C_{CO2} = \delta^{13}C_{DIC} + 23.644 - (9701.5 / [T + 273.15]) \quad \text{(Eq. 8)}$$

A warming of 5°C induces an increase in $\delta^{13}C$ of CO_2 of ~ 0.6‰ relative to DIC that needs to be accounted for when comparing coccolith carbon isotope signatures to an inorganic reference in culture or from coccoliths extracted from downcore sediments, as this thermodynamic feature is potentially able to explain $\delta^{18}O/\delta^{13}C$ co-variation, as recently demonstrated in calcareous dinoflagellates (Minoletti *et al.*, 2014). As temperature differently affects the carbon equilibrium constants of the distinct DIC species, investigating changes in $\delta^{13}C$ in culture may also represent a useful means of quantifying the relative HCO_3^- *versus* CO_2 assimilation in calcifying phytoplankton.

Summary

In the carbon isotope system, two primary factors drive the actual composition in coccolith calcite: the source of DIC acquired by the cell, and the effect of photosynthesis on the internal carbon pool. True $^{13}C/^{12}C$ fractionation in coccolith calcite is hidden by photosynthetic activity that depletes the intracellular DIC pool in ^{13}C. This also indicates that the choice of the culture set-up, and the concentration of cells at harvest, have significant artefactual effects on measured coccolith $\delta^{13}C$. Adopting a turbidostat and gentle resuspension of the cells may appear appropriate to diminish culture artefacts.

It is still undetermined whether this reservoir effect only jeopardises carbon isotope measurement from culture data, or if this process also operates in the natural environment via an alteration of carbon isotope of the DIC within the cell and/or in the boundary layer. Further chemostat work with efficient, but gentle, cell motions in the culture flask would help elucidating this point.

Oxygen isotope system

Overview

Oxygen isotope composition is probably the most applied coccolith-based proxy in Earth sciences. There is a strong temperature dependence on the magnitude of $^{18}O/^{16}O$ fractionation in calcite that palaeoceanographers utilise as a proxy for sea surface temperatures (SSTs). What makes this proxy imperfect, besides the vital effect, is that incorporation of ^{16}O *versus* ^{18}O into calcite is also dependent on the isotopic composition of seawater. The glacial-interglacial record during the Pleistocene primarily records fluctuations of seawater $\delta^{18}O$ due to ice volume change (Lisiecki & Raymo, 2005). Temperature changes are only a secondary contributor to the amplitude of the isotopic marine stages seen in the $\delta^{18}O$ signal in carbonate.

Published laboratory calibrations have been obtained by culturing different species under a range of temperatures compatible with growth and calcification. To date, all of the calibration curves have more or less similar slopes relative to equilibrium calcite and the vital effect is expressed by contrasting intersects (Fig. 3). Dudley *et al.* (1986) coined the terms "heavy group" and "light group" to describe the oxygen isotope behaviour of different coccolithophore species. This typology corresponds to the direction of the isotopic offset of coccoliths with respect to the composition of equilibrium calcite. To determine the latter ($\delta^{18}O_{equilibrium}$), and knowing the temperature of calcification and seawater $\delta^{18}O$ ($\delta^{18}O_{sw}$), we can use the formula derived from the work by Kim & O'Neil (1997) (Eq. 9).

$$\delta^{18}O_{equilibrium} = (0.0009 \times T^2) - (0.2468 \times T) + 3.7434 - (1.42 \times [pH - 7.8]) - 0.27 + \delta^{18}O_{sw} \quad \text{(Eq. 9)}$$

The factor of 1.42 in the intersect of the equation corresponds to the pH-dependence on $\delta^{18}O$ that operates via a concurrent change in the relative abundance between the DIC species in solution (Zeebe *et al.*, 2001). This pH effect has to be kept in mind when comparing results from batch experiments that have been conducted at different pH. The 0.27 coefficient is the last estimate used for the conversion from the V-SMOW used for seawater to V-PBD scale used for calcite (Hut, 1987). At $\delta^{18}O_{sw}$ = 0‰ and constant pH, this equation can be simplified if applied within the narrow range of temperature change compatible with coccolithophore tolerance (Eq. 10).

$$\delta^{18}O_{equilibrium} = -0.21 \times T + 2.78 \quad \text{(Eq. 10)}$$

The slope of the equation gives the sensitivity of the magnitude of calcite $\delta^{18}O_c$ change with temperature and hence, of the proxy. This parameter seems unaffected by the vital effect (Fig. 4).

Typology and coccolith assignment to an isotopic group

The "heavy group" is restricted to species of the Noelaerhabdaceae family, and all the species of this taxon belong to this group. This is the case for *Emiliania huxleyi*, *Gephyrocapsa oceanica* and *Crenalithus* (=*Reticulofenestra*) *sessilis* (Dudley *et al.*, 1986). All subsequent culture studies of these species have confirmed this isotopic behaviour (Ziveri *et al.*, 2003; Rickaby *et al.*, 2010; Moolna & Rickaby, 2012; Candelier, 2013; Hermoso *et al.*, 2014; Stevenson *et al.*, 2014).

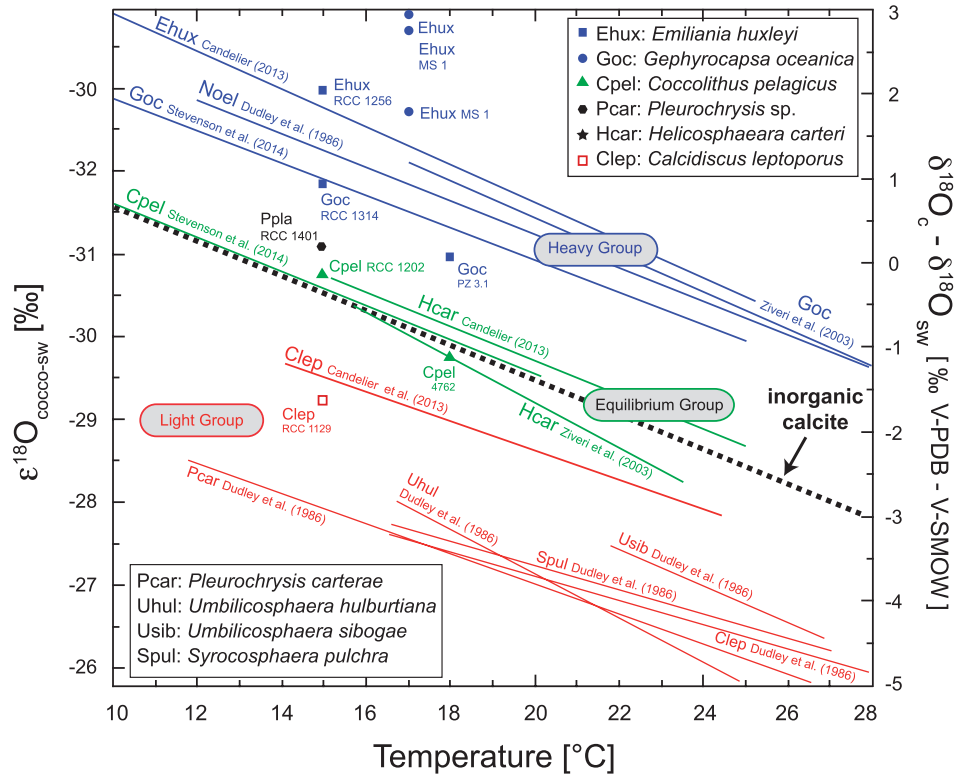


Fig. 4. Compilation of existing oxygen isotope compositions of cultured coccoliths under a wide range of temperatures. The vertical axis on the left reports fractionation coefficients. The axis on the right indicates the "$\delta^{18}O_{coccolith} - \delta^{18}O_{seawater}$" notation. The inorganic reference is calculated following the equation given in section 2 for pH 8.2 (derived from the work of Kim & O'Neil, 1997). Lines represents least square fits of published equations. Above this dotted bold line, coccoliths belong to an isotopic "heavy group" (*E. huxleyi* and *G. oceanica;* "Noel" comprises these two species and *Reticulofenestra sessilis; Dudley et al*., (1986)). Close to this line, coccoliths are part of an "equilibrium group" (*C. pelagicus* and *H. carteri*). Below the equilibrium line, coccoliths are assigned to an isotopically "light group" (*C. leptoporus*). Note that contrasting values have been reported between studies for *Pleurochrysis* sp. preventing its assignment to a group. References are given along each culture line. Individual measurements at 15°C are reported from Hermoso *et al*. (2014); *E. huxleyi* data points at 17°C are from Ziveri *et al.* (2003); those at 18°C (unmodified medium) come from Rickaby *et al.* (2010). The oxygen isotope composition of equilibrium calcite is calculated after the Eq. 9 in the text.

E. huxleyi always exhibits heavier oxygen isotope signatures with respect to *G. oceanica*. The isotopically "light group" comprises all species characterised by $\delta^{18}O$ signatures lower than equilibrium, such as *Calcidiscus leptoporus*, *Syracosphaera pulchra*, *Cricosphaera* (=*Pleurochrysis*) *carterae*, *Umbilicosphaera hulburtiana*, and *U. sibogae* (Dudley *et al*., 1986). Recently, Rickaby *et al*. (2010) and Stevenson *et al*. (2014) have shown that the large coccolithophore *Coccolithus pelagicus* produces calcite in near-equilibrium conditions.

The recent isotopic work by Candelier *et al*. (2013) on the genus *Calcidiscus* has confirmed the assignment of this taxon to the isotopic "light –

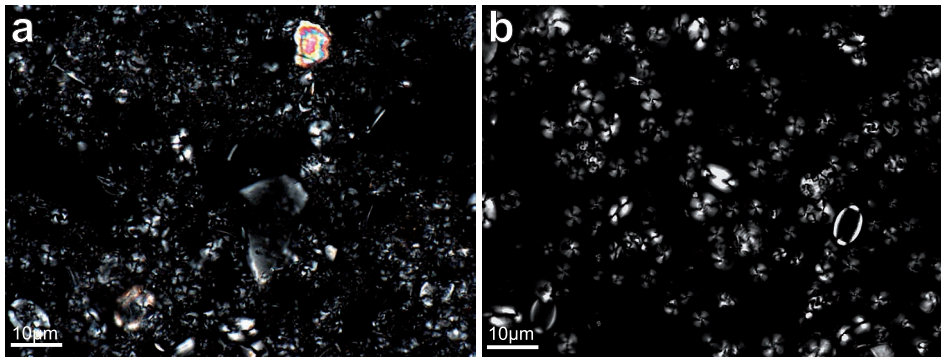


Fig. 5. Example of a *Calcidiscus* spp. assemblage microseparated from a North Atlantic Ocean core top sediment separated and isotopically analysed by Candelier *et al.* (2013) for the calibration of oxygen isotope in coccolith calcite. The microphotography a. shows the highly heterogeneous original coccolith assemblage; b. shows a near mono-specific fraction obtained by the Minoletti protocol (see Fig. 2).

group" with, however, significantly different (lower) fractionation factors than found by Dudley *et al.* (1986). Furthermore, it has been shown that the two main species of the taxon, *C. leptoporus* and *C. quadriperforatus*, as well as the medium and large morphotypes of the former, have indistinguishable $\delta^{18}O$ signatures. This work was the first to attempt calibration of $\delta^{18}O$ in coccolith calcite combining very dilute batch cultures and core top microseparated near-monospecific assemblages (Fig. 5). A good agreement was found between the two approaches. The cause of this substantial discrepancy between culture studies (1.3‰, corresponding to ~ 5°C; Fig. 4) remains unexplained.

At face value, offsets from equilibrium may correspond to the record of CO_2 ("heavy group") and CO_3^{2-} ("light group") in coccolith calcite, as equilibrium is calculated from a dominance (~ 90% of total DIC species) of HCO_3^- signature at pH around 8 (Fig. 4). The oxygen isotope system can be tentatively used as a tracer of which isotopic end-member of the DIC species is used for calcification at a given temperature. This indicates that parameters other than temperature – each of them representing a component of the vital effect – can influence oxygen isotope fractionation.

Heavy group

The dynamics of DIC turnover within the cell is key for the oxygen isotope composition of coccolith calcite in CO_2-utilising species. Amongst DIC species (aqueous CO_2 – $HCO3^-$ – CO_3^{2-}), CO_2 has particularly heavy (relatively positive $\delta^{18}O$) oxygen isotope signature (Usdowski *et al.*, 1991; Zeebe & Wolf-Gladrow, 2001). This indicates that in CO_2-utilising species such as most coccolithophores (Nimer & Merrett, 1996), the internal pool of DIC derives from a CO_2 source. This DIC ionic species is subsequently converted into HCO_3^- in the cell, but temporarily maintains the isotopically heavy $\delta^{18}O$ signature of CO_2. Indeed, a specificity of the oxygen isotope system, which is not operating for carbon, is that DIC ions are in constant equilibrium exchange with water molecules that represent an infinite oxygen reservoir. Total re-equilibration of the whole oxygen isotope system (H_2O – CO_2 – $HCO3^-$ – CO_3^{2-}) takes a matter of hours – on the order of 12 hours at 15°C (Zeebe and Gladrow, 2001). This

transient thermodynamic disequilibrium is eventually erased, and is not recorded in calcite if total re-equilibration is achieved prior to calcification (Hermoso *et al*., 2014). As a consequence, the heavy group to which the fast growing *E. huxleyi* and *G. oceanica* belong to, is probably the consequence of the partial record of CO_2 signature in calcite.

Candelier (2013) failed to find a temperature signal in Noelaerhabdaceae subfossil assemblages (mostly of *Gephyrocapsa* spp. and *Emiliania huxleyi*) microseparated from core top sediment samples. This calls into question the suitability of extrapolating culture findings to the geological record, even if very dilute cultures have confirmed that coccoliths of this family exhibit heavy $\delta^{18}O_c$ compared to equilibrium (Figs 1 & 2). This laboratory/natural environment discrepancy needs to be elucidated. This difference could tentatively be explained by light and nutrient-replete conditions imposed in cultures that may promote maximum physiological growth rates, and hence an artificially high record of the heavy isotopic signature of CO_2.

Equilibrium group

Coccoliths with $\delta^{18}O$ values close to equilibrium calcite are produced by relatively large cells such as *Coccolithus pelagicus*, *Helicosphaera carteri* and *Pleurochrysis placolithoides* (Fig. 4). It has to be noted that contrasting $\delta^{18}O$ values have been reported from different studies, but at very dilute growth conditions their near-equilibrium composition seems consistent and reproducible. In the case of *C. pelagicus*, the combination of a large cell geometry (limiting the passive diffusion of CO_2) and inefficient CCMs explain the very slow dynamics of the intracellular DIC pool. Under these circumstances, the DIC – H_2O oxygen isotope system is completely re-equilibrated when coccolithogenesis takes place, resulting in an equilibrium isotope composition. Surprisingly, *P. placolithoides* has been reported with efficient CCMs, its growth rate is relatively fast and it has a low PIC/POC ratio, suggesting a demand-to-supply of CO_2 that is satisfied in the cell. *P. placolithoides* would therefore be expected to belong to the isotopically heavy group. Only one single measurement exists beyond the dataset produced on *P. carterae* by Dudley & Goodney (1979) and Dudley *et al*. (1986), but it appears that its $\delta^{18}O$ is slightly higher than equilibrium (Fig. 4). One main difference with species of the heavy group is their large cell diameter. Another possible explanation is the expression of CCMs in this species leads to a dominant assimilation of HCO_3^- by the cell. Subsequently, as there is no ^{18}O-CO_2 disequilibrium affecting the intracellular DIC pool, coccoliths would record near-equilibrium isotopic composition. It is worth noting that the nature of the uptake (HCO_3^- *versus* CO_2) may also explain why *G. oceanica* is less shifted than *E. huxleyi* towards the "heavy group", as the former combines active HCO_3^- uptake and passive diffusion of CO_2 (Rickaby *et al*., 2010), while *E. huxleyi* only relies on passive diffusion of CO_2 under "normal" ambient DIC concentrations (Nimer & Merrett, 1996).

Light group

The carbonate ion has an equilibrium oxygen isotope composition lower than that of the bicarbonate ion. Calcification using a CO_3^{2-} – with its specific equilibrium composition – could then explain $\delta^{18}O$ values in calcite below equilibrium. In coccolithophores, the absence of transmembrane CO_3^{2-} transporters as part of the CCMs makes it difficult to explain the isotopic "light" group by a specific DIC uptake, as for the "heavy group" and CO_2. Other

mechanisms have to be sought. Kinetic effects, in the case of rapid calcification rate, could explain preferential incorporation of ^{16}O in the calcite lattice. To date, there is no direct measurement of this physiological parameter for coccolithophores. Comparing the speed of formation of one individual coccolith within the cell along with full characterisation of the carbon pool ([DIC] & pH) would enable constraining the physiological or thermodynamic causes behind the isotopic "light group", as they remain elusive (see section "***Insights from inorganic precipitation on oxygen isotope composition of calcite***").

Other parameters influencing $\delta^{18}O$ composition of coccolith calcite

The culture work by Rickaby *et al.* (2010) has thrown valuable light onto the ^{18}O vital effect of *Coccolithus pelagicus*. They showed that changing ambient DIC concentrations (from 1100 to 7800 mM kg^{-1}) and CO_2^{atm} (as pH was kept constant) have consequences on growth rate and on $\delta^{18}O$ (and $\delta^{13}C$) values (Fig. 6). In *C. pelagicus*, alleviated CO_2 limitation results in acceleration of growth rates and also induces higher $\delta^{18}O$ compositions in coccoliths. This ^{18}O enrichment is likely to originate from the same isotopic CO_2 imprint as that described for coccoliths of the "heavy" group. Similarly, increased $\delta^{13}C$ of *C. pelagicus* is the likely consequence of a more intense Rayleigh distillation driven by a higher intensity of POC production. In the same study, it was shown that $^{18}O/^{16}O$ apparent fractionation in *G. oceanica* was insensitive to ambient DIC levels (Fig. 4) probably because this species is not carbon limited at normal CO_2 levels, and increased carbon availability does not increase growth of this species. Hence, a powerful $\Delta\ \delta_{C.pelagicus} - \delta_{G.oceanica}$ proxy exists with insights into reconstructions of DIC concentrations that will help resolving problems associated with alkenone $\delta^{13}C$-based palaeo-CO_2 estimates raised by Zhang *et al.* (2013). Using a similar interspecies approach, $\Delta\ \delta_{C.leptoporus} - \delta_{G.oceanica}$ measurements can potentially

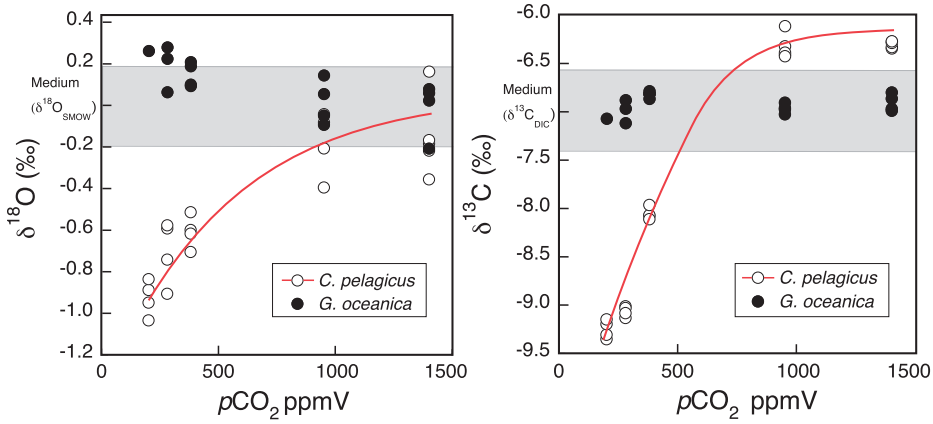


Fig. 6. Effect of ambient CO_2 concentration in the culture medium at constant pH on stable carbon (left panel) and oxygen (right panel) isotope composition for *Coccolithus pelagicus* (open symbols) and *Gephyrocapsa oceanica* (filled symbols). *G. oceanica* is insensitive to amendment of the medium with values within analytical errors. By contrast, there is substantial isotopic effect for the large and CO_2-limited species *C. pelagicus*. Alleviation of this limitation boosts photosynthesis leading to isotopic behaviour becoming similar to that of the "heavy group". Redrawn from Rickaby *et al.* (2010).

help us untangle the relative contribution of temperature and seawater $\delta^{18}O$ in geological fluctuations of oxygen isotope composition of the carbonate archive (Hermoso *et al*., 2014).

A carbonate ion effect has been suggested for *C. leptoporus* (Ziveri *et al*., 2012). This effect, previously documented on foraminifera, consists of a change in $\delta^{18}O$ with varying CO_3^{2-} concentrations in the culture medium. Indeed, the results presented indicate a negative linear relationship between $[CO_3^{2-}]$ and $\delta^{18}O$, i.e. increased carbonate ion concentrations are seen with more negative $\delta^{18}O$ values. The mode of DIC incorporation by coccolithophores, and marine algae in general, differs from that of foraminifera, with no CO_3^{2-} transporters being identified as part of CCMs. The implementation of the culture set up in the work by Ziveri *et al*. (2012) by addition of HCl/NaOH to reach the target CO_3^{2-} concentrations induced substantial changes to the entire chemistry of the DIC system and pH. Increased pH, as obtained by NaOH addition, is also accompanied by decreased aqueous CO_2 concentration, reducing the passive influx of DIC into the cell, and therefore, potentially limiting growth (data not published in the study). Although there is no ambiguity on a large effect of apparent fractionation of oxygen isotopes in the culture conditions *C. leptoporus* was exposed to, there are many factors that could contribute to this isotopic effect besides changes in $[CO_3^{2-}]$.

Insights from inorganic precipitation on oxygen isotope composition of calcite

In an inorganic precipitation system, calcification rate has significant influence (up to 1.5‰) on $\delta^{18}O$ values (Gabitov *et al*., 2012; Watkins *et al*., 2013). Little is known on this parameter in coccolithophores, which is complicated to measure directly. The few existing data come from average mass of calcite produced over the course of batch experiments (see Appendix 2 in Stoll *et al*., 2002). In the study by Taylor *et al*. (2007), calcification in *C. pelagicus* was filmed and the duration of coccolithogenesis measured. From a decalcified state, the formation of one single coccolith (representing ~ 150 pg of calcite; Young & Ziveri (2000)) took approximately 3 hours. This calcification rate is comparable to that of inorganically-precipitated calcite in the authoritative work by Kim and O'Neil (1997) that serves as an equilibrium reference for $\delta^{18}O$ values. There are two physiological parameters that may modulate calcification rates, and hence potentially drive oxygen isotope composition of coccoliths: the degree of saturation with respect to calcium (Ω_{CaCO3}) in the coccolith vesicle and the organic template involved in the biomineralising process.

The saturation state in the coccolith vesicle and pH are important parameters, yet are difficult to measure. They correspond to the imbalance between influx of Ca^{2+} and DIC into the coccolith vesicle and mineralisation, the latter representing the output of the biomineralising system. To date, only one study has constrained intracellular pH in coccolithophore cells (Anning *et al*., 1996). It has been reported that both *E. huxleyi* and *C. pelagicus* had similar pH (~ 7) at their site of calcification, ruling out a control of this parameter on the magnitude of ^{18}O fractionation. Considering that *C. pelagicus* precipitates calcite in near-equilibrium conditions for oxygen isotopes, and that *E. huxleyi* is ^{18}O-enriched with $\delta^{18}O$ values 3‰ higher, this offset would require a pH difference of more than 4 pH units – a hypothesis that is not tenable.

This relatively low measured pH compared to seawater values may appear surprising for a calcifying system. This feature results from the mode of biomineralisation of coccoliths that uses Ca^{2+}-binding acidic polysaccharides

(Westbroeck *et al*., 1984; Marin *et al*., 2014). It is worth noting that the catalytic role of the organic template on calcification may be accompanied with kinetic fractionation effects that still need to be explored. Localised pH increase also occurs during the H^+/Ca^{2+} exchange that promote calcification on this organic scaffolding. Such a phenomenon may record transient isotopic disequilibrium affecting the DIC pool at the initiation and during the course of coccolithogenesis.

Influence of the culture technique on $\delta^{18}O$ composition of coccoliths

There seems to be no influence on $\delta^{18}O$ values in cultured coccoliths during continuous batch cultures (Fig. 1). This observation has two main implications. First, discrepancies between culture experiments published by several authors cannot be explained by differences in culture set up, as claimed by Candelier *et al.* (2003) for *C. leptoporus*, and the significant offset they found with respect to the pioneered work by Dudley *et al.* (1986). Only few details are mentioned in the publication by Dudley *et al*. (1986) about the culture set up and the nature and preparation of the culture medium, preventing further assumption on the cause of obtained offsets (Fig. 1). Secondly, it is apparent that the significant pH drift of the culture medium has no apparent consequence on $\delta^{18}O$ values, suggesting a highly buffered intracellular environment. It has to be acknowledged that these two statements come from observations made on *G. oceanica*, and there is still a possibility that *C. leptoporus* has very distinct isotopic responses, especially with regard to its sensitivity to the carbonate ion effect.

Summary

All cultured coccolith species show a large temperature dependence on $\delta^{18}O$ values. Departures from equilibrium depend on a combination of equilibrium and kinetic fractionation processes that are combined in the concept of residence time of the DIC within the cell. We still have to explore potential modulations of oxygen isotope fractionation with growth rates, light, carbon, nutrient levels, especially if we want to ensure that culture findings in which all of these parameters are optimal are transferable to the geological record.

A full understanding of oxygen isotope fractionation will necessarily depend upon more physiological work to characterise pH and Ω_{CaCO3} values in the coccolith vesicle for a wide range of species and environmental conditions.

Carbonate clumped isotopes

Carbonate clumped isotope palaeothermometry (Δ_{47}) represents an emerging proxy in Earth sciences. Δ_{47} measurements are based on temperature-dependence of rare isotopes (^{13}C & ^{18}O) (e.g. Eiler, 2007). One of the advantages of this technique over the oxygen stable isotope proxy is that Δ_{47} of carbonate can generate temperature estimates without assumptions on the composition of seawater or on the mineralising fluid.

The work by Tripati *et al*. (2010) is the only study that has measured Δ_{47} composition of cultured coccoliths. These preliminary data, however, only comprise two unreplicated datapoints: *E. huxleyi* at 15°C and *C. pelagicus* ssp. *braarudii* at 10°C, and cultures were harvested at very dense cell concentrations. From these data, the authors suggested that there is no significant isotopic difference between coccolith Δ_{47} and foraminifera, and more broadly that these biogenic carbonates are devoid of a vital effect component. The lack of an apparent expression of a

vital effect in Δ_{47} composition in *E. huxleyi* that exhibits substantial physiological effects on oxygen isotopes reflects distinct dynamics and equilibrium constants in these isotopic systems (Hill *et al*., 2013). Further work is required to examine coccolith Δ_{47} signatures over a wide range of temperature and for more geologically relevant species. It would be useful to establish such empirical calibrations between temperature and Δ_{47} implemented in dilute batches, and examine a possible vital effect on Δ_{47} signatures in conditions mimicking the natural environment.

If it is confirmed that there is no interspecific differences, and that coccoliths precipitate calcite with Δ_{47} values close to predicted equilibrium (Katz *et al*., 2014), the use of the coccolith fraction (2 to 20 μm) for unravelling SSTs using this proxy would represent a valuable tool due to the relatively large quantity of material currently required to perform high-resolution Δ_{47} measurements (~ 5 mg of $CaCO_3$; Eiler, 2011). Application of this proxy will be of particular interest for periods during which sediments are devoid of foraminifera, such as the Palaeocene-Eocene Thermal Maximum (~ 55 Ma) due to problem with their preservation on the seafloor, and before their appearance in Late Jurassic times.

Preliminary culture data should be subsequently confirmed by a core top calibration to ensure there is no post-mortem obliteration of pristine Δ_{47} signature during export nor an effect accompanying early diagenesis where calcite particles are exposed to very cold temperature environments. For downcore investigation, it is also worth noting that the preservation of pristine Δ_{47} compositions in carbonate biominerals may represent a real challenge, if not a limitation of the proxy. Indeed, Δ_{47} are very sensitive to thermal diagenesis without coeval changes in $\delta^{18}O$ values (Henkes *et al*., 2014).

Calcium / Magnesium / Strontium

The common question that has led to the study of the non-traditional metal isotope systems in coccolith calcite was mainly concerned with the development of a palaeo-temperature proxy (Fig. 7). Lemarchand *et al*. (2004) found a temperature dependence of fractionation of calcium isotopes in inorganic experiments. Only preliminary measurements from cultured coccoliths have been made on calcium and calcium-substituted isotopes (Fig. 4). No core-top or downcore investigations of the systematics of these isotopic systems in coccoliths have been conducted yet. It seems that one major fractionation step of these cations occurs with the dehydration of the molecules accompanying transmembrane transport and incorporation into the cell and subsequently into the coccolith vesicle (Gussone *et al*., 2006; Stevenson *et al*., 2014). It has been found that calcium, magnesium and stable strontium isotopic compositions reflects to some extent the temperature of calcification, but there is an unresolved ambiguity as to whether these isotopes reflect temperature or growth rates – these two parameters being linked.

In *Calcidiscus leptoporus*, the expression of a carbonate ion effect (as on $\delta^{18}O$ values; Ziveri *et al*., 2012) also seems to be at play in $\delta^{44}Ca$ (Gussone *et al*., 2007), providing compelling evidence that calcification in this species may occur in a highly alkaline environment.

Constraining calcium isotopes would enable assessment of coccolith calcification rate by analogy to inorganic precipitation for which it has been shown that a large kinetic effect leading to light isotopes being more incorporated in a fast growing calcite crystal (DePaolo, 2011; Reynard *et al*., 2011).

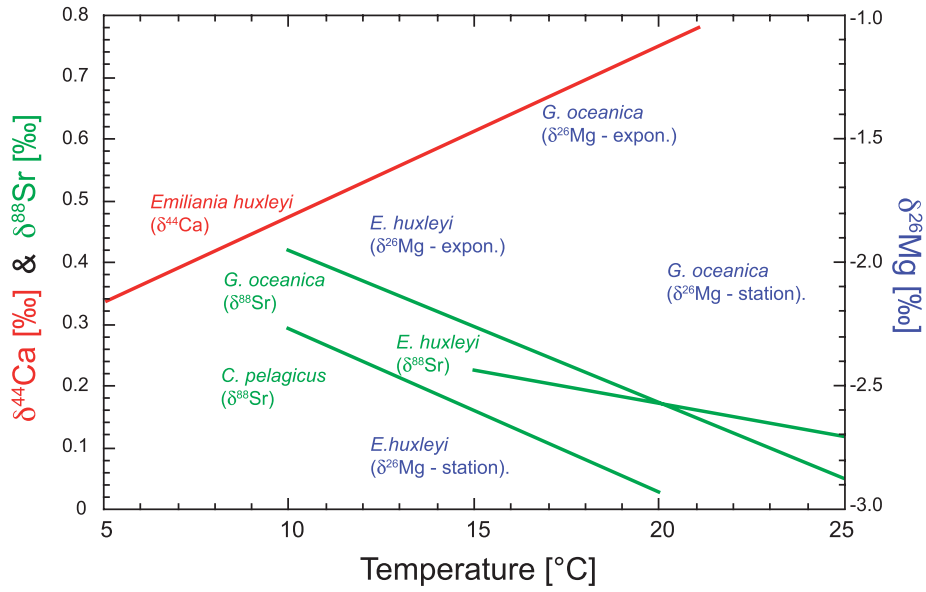


Fig. 7. Compilation of metal non-traditional isotope systems that have been measured on cultured coccolith calcite. Overall, there is an increase in growth rate with temperature. The co-variation of temperature and division rates makes interpretation of data complicated with regard to which parameters drive isotopic fractionation. Further work is needed to untangle the effect of each parameter. The magnesium isotope data significantly differ from measurements from material grown during the exponential and stationary phases. References: Calcium from Gussone *et al.* (2006); Magnesium from Ra et *al*. (2010); and Stable strontium from Stevenson *et al.* (2014).

Boron

No attempt to measure coccolith $\delta^{11}B$ composition, either from cultures or from sediments, has been made yet. Boron isotope signatures represent a promising proxy in palaeoceanography as they are supposed to reflect pH via incorporation of borate ions relative to boric acid into calcite. Both boron species have distinct equilibrium $\delta^{11}B$ composition, and their relative abundance is a function of pH.

The study by Stoll *et al*. (2012) has measured boron elemental ratios in coccolith calcite on *Emiliania huxleyi* and *Coccolithus pelagicus* with a view to constraining DIC concentration and pH at the site of calcification. Boron isotopes measurements of coccoliths may help resolving the ambiguous response of a pH and/or DIC control on elemental boron (B/Ca) incorporation into calcite.

The validation of the boron isotope proxy on coccolith material would first require determining whether amendment of external pH is seen intracellularly in $\delta^{11}B$ of coccoliths. Longer term, generating palaeo-pH data along with palaeo-CO_2 estimates obtained from alkenone carbon isotopes (Pagani, 2002) would permit insightful characterisation of the status of the carbonate ion chemistry at the surface waters in the past.

$$\varepsilon p\ [‰] = (\delta^{13}C_{CO2aq} - \delta^{13}C_{org}) / (1 + [\delta^{13}C_{org} / 1000]) \qquad \text{(Eq. 11)}$$

The magnitude of fractionation associated with photosynthesis in marine algae has been found to depend on the size of the carbon pool with higher εp values with a larger size of reservoir (kinetic effect). The calculation of $\delta^{13}C_{CO2aq}$ relies on a number of assumptions, and is tentatively inferred from foraminiferal $\delta^{13}C$ in downcore studies (Pagani, 2002). Not only would measurements of $\delta^{13}C_{Noelaerhabcacea}$ enable isotopic characterisation of the intracellular carbon pool, but also $\delta^{11}B_{Noelaerhabcacea}$ would allow constraining of the transfer function linking the cascade of biogeochemical and climate relevant parameters: $\delta^{13}C$ of the alkenones – εp – $[CO_{2aq}]$ – CO_2^{atm} (Eq. 11).

AN EVOLUTIONARY PERSPERCTIVE ON THE VITAL EFFECT

Emergence of the CCMs in coccolithophores

Empirical calibrations of the isotopes in coccolith calcite through laboratory cultures have utilised modern species. Hence, this approach aiming to produce vital effect-free isotopic signals from calcareous nannofossils heavily rely on the Uniformitarianism principle. Core top calibrations and analyses of "subfossils" enable investigation of population-scaled assemblages. If we want to apply fractionation coefficients accounting for the physiological effect on stable isotope composition in a downcore approach, we need to make the assumption that a considered species, or even its close relatives, have had similar isotopic behaviour over the last few kyr or even Myr.

Bolton *et al.* (2012) and subsequently Bolton & Stoll (2013) have suggested an evolutionary concept of the vital effect. They have linked two concepts: the appearance of interspecies vital effects (and potentially "absolute" vital effects) and CCMs. They have suggested that before the Late Miocene sub-epoch, when CO_2^{atm} were substantially high compared to present-day, all coccolithophore species had similar carbon isotope signatures because CCMs were not operating as the photosynthetic demand for carbon was satisfied. After a threshold induced by declining CO_2^{atm} concentrations, interspecies vital effects appeared. At face value, this hypothesis would indicate that during the vast majority of coccolithophore stratigraphic extension (from the Mesozoic to the beginning of the Cenozoic – approximately between 190 and 5 Ma), all coccoliths have been produced with similar vital effects. In turn, this suggests that measured geochemical differences between coccoliths species originate only from ecological specificities (seasonality, calcification depth). This important discovery has major implications for palaeoceanographic studies. Although the observations and arguments made in this study appear to be robust, it would be necessary to undertake further investigation of interspecific vital effect in the geological history to prove this hypothesis. Hermoso *et al.* (2009b) found significant interspecies variability in both $\delta^{13}C$ and $\delta^{18}O$ composition of coccoliths deposited onto the Onto-Java Plateau during the Middle Miocene (~ 15 – 18 Ma), hence challenging the stratigraphic position of this putative threshold.

Change in coccolithophore cell size

Cell size, via the surface-to-volume ratio, has a strong influence on DIC assimilation by coccolithophores, growth dynamics, and vital effect. Small cells,

such as *E. huxleyi*, have a high degree of passive CO_2 diffusion owing to their high surface-to-volume ratio. Under ambient CO_2 limitation recreated in the laboratory, some strains of this species have the ability to excrete carbonic anhydrase (catalysing the dehydration of the abundant HCO_3^- into aqueous CO_2) in the periplasmic environment to promote passive diffusion of CO_2 into the cell (Nimer & Merrett, 1996). As a result, in modern-day settings, *E. huxleyi* appears not to be CO_2 limited, explaining its prolific abundance in the oceans. For both carbon and oxygen isotopes, *E. huxleyi* has the strongest vital effects compared to other extent species regardless of whether or not this corresponds to true magnitudes of fractionation.

The long-term reduction in cell, coccosphere and coccolith size of the Noelaerhabdaceae family over the Cenozoic is thought to be the consequence of declining CO_2 concentrations – a component of the CCMs in this lineage. Indeed, reduced size makes cells more permeable to CO_2. The foremost representative of this family during the Cenozoic, *Reticulofenestra* sp., had significantly larger cell, coccosphere and coccolith sizes compared to its extant close relative, *E. huxleyi*, and even bigger than *G. oceanica*. Based on coccolith size, it is likely that some early reticulofenestrid cells reached *C. pelagicus* diameter, i.e. up to 20 μm in diameter (Henderiks & Pagani, 2008). Even if the extant *Reticulofenestra sessilis* has proven to be part of the isotopic "heavy group" (Dudley *et al*., 1986), it is too speculative to apply a fractionation coefficient determined for *E. huxleyi* or *G. oceanica* to the larger *Reticulofenestra* sp. for its entire stratigraphic range. Such an assumption would require further characterisation of cell size using coccolith size (Henderiks, 2008), and consideration of prevailing settings, such as nutrient, light, and DIC, that, taken together, may influence growth and hence fractionation. It will probably never be possible to directly characterise palaeo-CCMs by exploring the fossil record. At present, the uncertainty in deriving SST from a reticulofenestrid assemblage in the Neogene is substantial (~ 12°C), as it could span from no vital effect, as is the case for CO_2 limitation in *C. pelagicus*, to a + 3‰ offset from $\delta^{18}O$ equilibrium if cells were as carbon-replete and successful as *E. huxleyi*.

Change in ecology of coccolithophores species

Contrary to the Noelaerhabdaceae coccoliths, *Coccolithus pelagicus* has not significantly changed its size with the Cenozoic decline of CO_2^{atm} (Henderiks & Rickaby, 2007). The polewards migration of *Coccolithus pelagicus* was its likely response to progressive CO_2 limitation in order to maintain sufficient CO_2 levels for photosynthesis. This ecological and biogeochemical adaptation illustrates the problem of the Uniformitarianism principle. Before the migration of *C. pelagicus*, this relatively successful species (with an abundant fossil record over the Cenozoic) was used to thrive in warm equatorial waters. This species is now nearly restricted to high latitude and upwelling regions where [DIC] are more elevated. Hence, one may question the effect of this feature on the magnitude of the vital effect. The work by Rickaby *et al*. (2010) has highlighted the significant effect of alleviating CO_2 limitation on the vital effect for both the carbon and oxygen isotope systems (Fig. 6). This change in stable isotope composition may be the effect of the modulation in growth rate (Hermoso *et al*., 2014), and prevents application of a unique fractionation coefficient to translate oxygen isotope compositions into temperature estimates for this species extracted from downcore sediments. It appears likely that during most of the Cenozoic, this species produced coccoliths with isotopically heavier $\delta^{18}O$ with respect to present-day. Exploiting such a

modulation of the expression of the vital effect can be sought with a view to unravelling palaeo-CO_2 concentrations by coeval measurements of other coccolith species or surface-dwelling foraminifera whose $\delta^{18}O$ are insensitive to [$CO2_{aq}$].

FUTURE RESEARCH AVENUES

Vital effects in coccolith calcite have been regarded as a black box for the last three decades. Recent and ongoing biogeochemical developments aim to identify the mechanisms responsible for physiological effects on stable isotope composition of coccolith calcite. These works indicate that the vital effect can be replicated in laboratory cultures, and constrained. They further show that this important factor of coccolith isotope fractionation is mediated by the physiology, but is ultimately under environmental control. This latter control is first expressed by the modulation of the physiology and therefore of the vital effect, and is subsequently influencing thermodynamic inorganic reactions leading to calcification. The expression of the vital effect in coccolith calcite significantly differs from what happens in foraminifera and coral calcite owing to predominant CO_2 assimilation by the cell, and a unique degree of coupling between calcification and photosynthesis.

Coccolithophore algae produce both calcite and alkenones that are preserved in sediments, and a coupled organic/inorganic downcore investigation through the Cenozoic represents a promising way to reconcile the data and overcome current limitations in palaeoclimate reconstruction at various timescales. Progresses in the refinement of alkenone isotope transfer functions would represent valuable proxy development in palaeoceanography. Utilising the coccolith record would help overcoming current uncertainties associated with reconstruction of alkenone isotope fractionation factors (Zhang *et al*., 2013). The unsaturation index of alkenones ($U_k^{37'}$) is a proxy for SSTs (Prahl *et al*., 2000). Although this tool does not represent a coccolith-based proxy, the opportunity to have access to two temperature proxies by estimates from the same phytoplankton producers would be useful to better unravel paleoclimates using an organic – inorganic approach.

Preliminary work on the utilisation of $\delta^{13}C$ of the organic template encapsulated inside the coccoliths has been recently carried out (Lee *et al*., 2014). Measurements of $\delta^{13}C_{carb}$ of the coccoliths and $\delta^{13}C_{CAP}$ (coccolith-associated polysaccharides) may therefore represent a valuable and unprecedented palaeo-CO_2 barometer. From an isotopic perspective, it would be interesting to seek a vital effect component (kinetic effects) originating from the concentration of CAP in the coccoliths (Kayano *et al*., 2011). Developing this proxy may transcend the current limitations in the use of the $\delta^{13}C$ of alkenones (εp), owing to the lack of an inorganic reference of the rather limited temporal applicability of the tool in the geological history (Pagani *et al*., 2005; Zhang *et al*., 2013).

A cross species approach would take advantage of the distinct species-specific responses of coccolithophores (and coccolith geochemistry) to changes in environmental conditions. To date, this approach includes a palaeo-DIC and a palaeo-$\delta^{18}O_{sw}$ proxy (Rickaby *et al*., 2010; Hermoso *et al*., 2014). Further culture and field investigations of coccolith fractionation, especially for non-traditional

isotopes, will refine our understanding of the mechanism behind the vital effect and establish new geochemical tools of interest for palaeoceanographic studies.

An effort to develop existing protocols for extracting coccoliths from sediments is now required to be able to perform targeted analyses of coccoliths more routinely. There is room for improvement of these techniques. In particular, it appears necessary to proceed to some methodological development with a view to decreasing coccolith fragmentation due to ultrasonic treatment or use of solutions with $\Omega_{CaCO3} << 1$ – even neutralised – and to reduce the time-consuming nature of the process by implementing semi-automatic steps (addition of water level sensors in the column, automatic addition of suspension to filter, *etc*).

CONCLUSIONS

Most of the vital effect in coccolith calcite can be explained by a combination of equilibrium isotope fractionation related to the nature of the DIC uptake by the cell, to which is superimposed kinetic effects occurring during transmembrane transport within the cell's compartments and eventually during biomineralisation. There is an additional Raleigh distillation process due to photosynthetic carbon fixation that dictates carbon isotope composition in coccoliths, and to some extent obliterates the true $^{12}C/^{13}C$ fractionation especially in fast growing species with high photosynthetic rates such as *E. huxleyi*.

The majority of cultures that have been undertaken have studied *E. huxleyi*. The choice of this model organism probably pertains to the relative ease to culture it and to its relevance for the fate of the biological carbonate pump during the Anthropogene. However, this taxon has only a limited geological record and there are questions about whether findings for this species can be transferred to older species, even within the Noelaerhabdaceae family. We need to gain a better understanding of fractionation processes for a wide of geologically relevant coccolith species.

The mode of culture strategies in the laboratory has a large influence on isotopic composition of coccoliths leading to biases that correspond to measurements of apparent fractionation factors. Overall, revised calibrations tend to indicate more restricted interspecific vital effects than previously thought. Semi-continuous or chemostat implementations have to be favoured for future calibration efforts.

An evolutionary perspective on the vital effect can be achieved though an interspecific or intergroup (foraminifera or calcareous dinoflagellate) approach. Exploring the nannofossil record would require estimates of cell geometry and palaeo-growth rate to assess the likely offset from equilibrium composition of extinct species.

There has been a particular effort to explore the possibility of the use of isotopic proxies to derive palaeoenvironment, such as sea surface temperatures via oxygen isotope calibration and downcore measurements. Other components of the environmental settings have yet to be explored using coccoliths, especially by applying a cross-species strategy.

The development of non-traditional isotopes will not only provide new insights into coccolith-derived palaeoceanographic reconstructions, but will also help better understanding the carbon and oxygen isotopes systems.

As it is still a challenge to extract coccolith fractions from sediments, the application of these proxies can be limited. This justifies the need to improve current methodologies for microseparating coccoliths at the purest species-specific level.

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