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Biogeosciences Editorial Board

Dear Professor Bijma,

We thank the three Referees for their insightful comments and your considered guidance for the revisions. As you will see in the cumulative point-by-point response, we have dealt with all the points arisen from peer-review and welcomed most of the comments and suggestions that have substantially improved our manuscript. Importantly, we feel that the problem linked with "the CO_2 *vs* HCO_3^- controversy" quoted by Referee#2 has been resolved by altering the text to reflect carbon (DIC) availability generally.

Following your own recommendation and that of Referee#2 during the Interactive Discussion, we have removed the sections and figures presenting and discussing the *DCUt* index. *Section 3.3*. has been removed; *Section 4.2* has been amended and we now discuss the magnitude of the vital effect in the context of ambient total DIC concentrations. We do not explicitly, nor implicitly state that CO_2 is the prime source of carbon used for calcification in coccolithophores in our revisions, and instead adopt a pure empirical calibration by considering DIC levels. The manuscript now focuses on the palaeoceanographic implications of this work by shortening the biogeochemical discussion (as suggested by Referee#3). Importantly, we do not feel that these changes have removed any substance from our work, at least from a geological perspective, and the data can be used to better interpret fossil coccolith isotopic signals (and bulk carbonate!) from the sedimentological record, as it was our primary aim.

Last, we apologise for not providing the raw data with our original submission, a table with all the collected dataset (media and calcite) used for this study has been prepared and will be submitted with the revised manuscript.

We thank you in advance for your consideration and we look forward to hearing from you.

Yours sincerely,

Dr Michael Hermoso and coauthors

Referee#1 (Anonymous)

The study: "Vanishing coccolith vital effects with alleviated CO2 limitation“ by Hermoso and co-workers contains highly interesting results on growth, and stable carbon/oxygen isotope fractionation in coccoliths of four different coccolithophore species. The laboratory work makes a very good impression although I cannot really comment on the isotope methodology. I have, however, one major and one minor concern with the data interpretation. I will try to explain these concerns in the following.

Major concern: A core parameter in your study is DCUt. To calculate this parameter you assume that “passive influx of CO2 constitutes the only source of carbon to the cell”. I have very strong concerns with this assumption (which seems to be central to many of your interpretations and hypothesis) and worry that it is not valid. You underline this assumption with studies by Sekino and Shiraiwa (1994) and Kottmeier et al., (2014).

However, Sekino and Shiraiwa (1994) stated in the abstract that “HCO3- was utilized mainly for production of CaCO3 and accumulation of internal inorganic carbon” which contradicts your assumption. Kottmeier et al. (2014) indeed showed that CO2 is the dominant DIC source under high DIC but this finding is only true for photosynthesis. Kottmeier et al., (2014) did not investigate the carbon source for calcification.

Furthermore, there are a large number of studies with different methodological approaches which have shown that HCO3- is a (or even the) key source ion for photosynthesis (e.g. Rost et al., 2003, 2006; Schulz et al., 2007) and calcification (e.g. Sikes et al., 1980, Nimer et al., 1993, Buitenhuis et al., 1999, Bach et al., 2013).

Please clarify this issue because if this assumption is not true then DCUt cannot be interpreted in the way you do in this paper.

(Please have a special look on lines 22-27 on page 15846, lines 23-29 on page 15849, and lines 17-18 on page 15855.)

Authors’s response: See previous answers on this specific point in the Interactive Discussion, and also how we have dealt with this problem in our introductory Revision Notes. We do not state that calcification originates from a CO_2 substrate (anymore). We have removed references to the *DCUt* index (explicitly using ambient [$CO_{2\ aq}$] concentrations) from the revised manuscript. In particular, the paragraphs mentioned by the Referee have been totally reformulated to avoid any confusion in the use of this “index”.

- Page 15846 lines 22-27

“*Assuming that the passive influx of CO_2 constitutes the only source of carbon to the cell,…*” has been **removed**.

“*We are aware of no evidence for increased HCO_3^- assimilation in coccolithophores under high CO_2 environments, so we assume prominent CO_2 influx at high DIC, consistent with the work of Kottmeier et al. (2014) and Hermoso (2015).*” has been **removed**.

- Page 15849 lines 23-29

The sentences "*We emphasise that our understanding of the internal carbon pool build-up favours a preponderant CO_2 assimilation by phytoplanktonic calcifiers and that both pathways use a common internal carbon pool (Sekino and Shiraiwa, 1994; Bolton and Stoll, 2013; Hermoso et al., 2014; Kottmeier et al., 2014). Hence, the assumption that calcification utilises bicarbonate ions transported from the extracellular environment to the coccolith vesicle with no influence from photosynthetic carbon fixation conflicts with many physiological and isotopic evidence.*" have been **removed**.

- Page 15855 lines 17-18

"*A "reverse" approach using the present calibration utilising the magnitude of the vital effect, appears possible to derive DCUt estimates that can be, in turn, linked to [$CO_{2\ aq}$] concentrations*". – sentence **deleted**.

Minor concern: DIC concentrations in the highest treatment were ~12000 µmol/kg. When I calculate Omega_calcite for this concentration (assuming pH 8.2 (pH scale missing! See comment 4), S=35 (not given, why?), T=15, K1/K2 by Mehrbach et al.1973 refitted by Dickson and Millero 1987) I get values of 26 (pH on free scale) or even 30 (pH on total scale). At such high Omega_calcite values there is a high potential of inorganic CaCO3 precipitation. Could this interfere with your results? And to some extent explain the absence of vital effects at high DIC? I noticed that you seem to address this issue at the beginning of section 4.1 but I did not understand your argumentation here.

Authors's response: The pH scale used is the Total Scale (now added in the ms). Salinity was 33 (information now given). In our medium, the addition of chelators such as EDTA and the presence of relatively elevated phosphate content prevent spontaneous precipitation in the culture medium We did not observe any evidence of inorganic calcite / aragonite precipitation either in the form of spines, orthorhombic crystals or overgrowths on coccolith calcite under the SEM. The mass of such inorganic calcite would need to be substantial to "homogenise" the isotopic signatures of coccoliths of the heavy (as *E. huxleyi*) and light (as *C. leptoporus*) groups.

Specific comments:

1) Page 15838 line 13: What do you mean by "primarily CO2"? Changing DIC at pH 8.2 primarily affects HCO3-.

Authors's response: "*primarily CO2*" has been **removed**.

2) Page 15838 line 17: The term "static vs. dynamic" is unclear in this context (at least for the reader not experienced with isotope geochemistry and vital effects).

Authors's response: This now reads: "…whether the vital effect in coccolith calcite remains constant for a given species or changes with the environment".

3) Page 15840 line 6: Perhaps a question which is a bit difficult to answer but do you expect that there is an effect of N2 purging on cell physiology? I mean, you effectively removed O2 and all other trace gases as well. I wonder if this makes a difference to the cells. (Since your growth rates are fine I don't think it does but I am just wondering.)

Authors's response: This is indeed a tricky question, but considering the very fast dynamics of photosynthetic carbon fixation in the cell and consecutive oxygen liberation, it is unlikely that such effects have influenced our results beyond the first minutes of the bioassays.

4) Page 15840 line 11. Please give the pH scale. This is absolutely essential in carbonate chemistry experiments.

Authors's response: This has been done (Total Scale).

5) Page 15840 lines 14-15. What do you mean by successive alterations of the carbonate chemistry. Please try to be less cryptic.

Authors's response: We refer to successive additions of HCl, $NaHCO_3$ and NaOH used to reach targeted DIC and pH values. This has been **added in text**.

6) Page 15846 line 25. Bach et al., 2014 does not exist. Do you mean 2013 or 2015?

Authors's response: We apologise for the confusion: "2014" has been **replaced** by "2013".

7) Page 15850 lines 13ff. Langer et al., (2009) only showed this for a much narrower range of carbonate chemistry conditions. I doubt that no changes in PIC/POC would occur in your experiment because your DIC range is huge.

Authors's response: According to Referee#2's suggestion the analogy with the work by Langer has been removed, and **replaced** by the more suitable study by Bach et al. (2013).

8) Page 15851 line 11ff. More recent results showed that another strain of C. pelagicus changes PIC/POC in response to changing carbonate chemistry (Bach et al., 2015).

Authors's response: Reference added.

9) Page 1854 Lines 18-21. I wonder: Isn't it a bit too optimistic to make this suggestion based on the current evidence?

Authors's response: We regard this as a fact, and this research avenue is also illustrated by the ongoing effort to explore the geochemistry of the coccoliths in the geological record (e.g. Bolton et al., 2012, amongst others). Nevertheless, we have **replaced** *levels* by *estimates* to slightly tone this down.

10) Page 15855 Lines 1f. This would only work if coccolith size is bound to cell size. However, there are also very large species with very small coccoliths (e.g. Pleurochrysis carterae).

Authors's response: This is true. *Pleurochrysis* spp. has no existence in the geological record, and for other species, the works by Henderiks provided reliable correlation between coccolith, coccosphere and cell size (Henderiks and Rickaby, 2007; Henderiks, 2008; Henderiks and Pagani 2008). Therefore, this possibility is feasible and ought to be further explored combining geochemical and morphological analyses of sedimentary coccoliths in the geological record.

I hope my comments help to improve the manuscript further.

Referee#2 (Lena-Maria Holtz)

General comments

Summary: The authors of the study cultured four different coccolithophore strains, each at six different carbonate systems, and measured the corresponding $\delta^{13}C$ and δ18O values of the coccoliths. Cells were cultured at densities that were too low to determine particulate inorganic and organic carbon (PIC and POC, respectively) per cell. All six carbonate systems have the same pH value, but different dissolved inorganic carbon concentrations (DIC): 2, 4, 6, 8, 10, and 12mmol·L−1. Measured δ13C and δ18O values are then plotted over DIC (or [CO_2]). A so-called carbon usage index (DCUt) is introduced (after Rau et al. [1] and Bidigare et al. [2]) and correlated to δ13C and δ18O values. Then, the authors try to interpret found correlations for δ13C and δ18O data from a mechanistic viewpoint.
One of my major concerns (complete list below) with the presented work is that the authors persistently argue that external CO_2 was the prime carbon source of calcite. This is against all experimental evidence (and also against their own presented data set). The authors know the relevant literature (I listet some of it in an earlier review for them and they cite some of it – for different aspects though). After reading the passage on page 15849 lines 23-end[1], I understood why they think that way.
1 *"We emphasise that our understanding of the internal carbon pool build-up favours a preponderant CO2 assimilation by phytoplanktonic calcifiers and that both pathways use a common internal carbon pool (Sekino and Shiraiwa, 1994; Bolton and Stoll, 2013; Hermoso et al., 2014; Kottmeier et al., 2014). Hence, the assumption that calcification utilises bicarbonate ions transported from the extracellular environment to the coccolith vesicle with no influence from photosynthetic carbon fixation conflicts with many physiological and isotopic evidence."*

They do not believe that the prime carbon source for calcite is HCO_3^- and that the isotopic signal in calcite is influenced by photosynthetic carbon fixation at the same time. I admit that this apparent controversity is difficult to think through just by means of a human brain. A computer-based model can help here. The symbiont-bearing foram model of Zeebe et al. [6] for instance faces a similar issue – with external (not internal) symbionts though – and also finds the carbon signal in calcite influenced by symbiont activity, although symbionts use CO2 and calcite is precipitated from CO32−. One thing that is essential to have in mind when aiming at understanding measured carbon isotopic signals is that internal carbonate systems of living cells are out of chemical equilibrium [8]. Furthermore, it is important to have in mind that there are "two carbonate systems" (12C and 13C) the reactions of which occur in parallel [7].
The presented data sets, however, show that HCO_3^- is the prime external carbon source of calcite in all four cocco species: The authors present the isotopic data as if HCO_3^- was presumed to be the prime carbon source of calcite:
$\delta^{13}C_{calcite}$ - $\delta^{13}C_{DIC}$ (where $\delta^{13}C_{DIC}$ d δ13C HCO_3^-) is presented and not $\delta^{13}C_{calcite}$ - $\delta^{13}C_{CO2}$.

Authors's response: The expression of this offset is justified by: i/ the fact that the inorganic calcite is precipitated from the relative proportion of the DIC species (the "S" of Zeebe), therefore primarily from HCO_3^- at the considered pH; and ii/ to conform to the palaeoceanographic usage (e.g. works by Spero; Ziveri and others). This is now even more

justified, as we do not make any *a priori* hypothesis on which DIC substrate is incorporated by the cell in the revised ms.

δ13Ccalcite - $\delta 13C_{DIC}$ does not deviate strongly from 0. $\delta 13C_{calcite}$ - $\delta 13C_{CO2}$, in contrast, would (not shown) strongly exceed zero. It hence follows that calcite was most likely built from external HCO_3^- and not CO2.

Authors's response: The difficulty here is to detangle true fractionation and apparent fractionation in coccolith calcite. *E. huxleyi* (the cell with the highest photosynthetic rates relative to calcification rates) calcite $\delta^{13}C$ values can be substantially high relative to inorganic calcite (2-3 ‰ higher than a HCO_3^- source). Indeed, this implies that $\delta^{13}C$ coccolith values are even more significantly shifted towards positive composition relative to CO_2. Once again in our newly revised manuscript, we do not assume a sole assimilation of CO_2 in our revisions, but rather develop an empirical calibration, hence we use $\delta^{13}C_{DIC}$ as the baseline from which we express the coccolith ^{13}C vital effect.

Major concerns

The heart piece of the work, i.e. the data set, is not presented.

Authors's response: A table presenting the collected data has been added within the ms.

Furthermore, no error bars (how many replicates?) are presented in the figures.

Authors's response: The error bars on coccolith stable isotope compositions would be smaller than the symbols on the figures. Two culture replicates were performed (information added in text).

Tables that list carbonate systems and results should be given.

Authors's response: Done.

Carbonate systems are manipulated unconventionally, why a presentation of the carbonate systems is even more important than usually. The authors mention a publication on their own website (data are not yet accessible). Since the data are the actual output of the work, I am of the opinion that they belong to the article.

Authors's response: Once again, the table will be fully part of the paper.

An (in my opinion) odd interpretation of a carbon usage index DCUt.

Authors's response: See previous discussion with Referee#1 and 2 during the Interactive Discussion Phase on this matter, and our revision notes at the beginning of this rebuttal letter. This index has been removed from our revisions.

Misinterpretation of some cited literature (see also referee 1).

Authors's response: This has been corrected, as e.g. the Sekino and Kottmeier papers now do not indicate the use of CO_2 for calcification.

I am not sure, but the correlation between δ13C and δ18O and this DCUt index (calculated from [CO2]) might originate from the correlation between δ13C and δ18O and [CO2] (latter correlation in most cases higher). This should be tested statistically.

Authors's response: See graphs provided during the Interactive Discussion. Most of the correlations indeed came from DIC (CO2) concentration. The old figures 3 have been **removed**.

I am of the opinion that the title statement "vanishing coccolith vital effects with alleviated CO2" is misleading and does not follow from the presented work.

Authors's response: We have change "CO_2" for "carbon" to account for the change made in the manuscript.

I am of the opinion that a profound discussion on cellular carbon fluxes when just having δ13C and δ18O data (without the corresponding data of the organic phase or particulate organic and inorganic carbon fixation rates) is not possible.

Authors's response: Yes, we fully agree. See response in the Interactive Discussion, and response on this point already made above.

Some specific comments

p. 15836
l. 5/6: "Under high DIC ...": (i) inorganic values were not measured, just calculated and may deviate from the calculation. However, this is not the point. (ii) "lacked any offset" is a very optimistic expression (compare data at 12mmol·L−1 with calculated inorganic value). (iii) since your regression lines (Fig. 2) are linear and do not approach the calculated value for inorganic calcite asymptotically, I am of the opinion that you cannot conclude that it "lacks any offset with inorganic calcite". What would, for instance, be the corresponding values at 14mmol·L−1 for E. huxleyi or C. pelagicus?

Authors's response: This is true. This sentence has been reformulated: "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 (present-day) DIC concentrations."

l. 15-18: I think you should discuss in which respect the established carbonate systems resemble those of (which?) geological times.

Authors's response: This would require precise knowledge of many natural environment parameters (temperature, salinity, pH, omega calcite etc….) that nobody knows with enough accuracy. These parameters are the quest of palaeoceanographers! We performed our experiments at one temperature, one pH, hence, it is just impossible to draw a parallel (especially at a global scale) between experimental conditions and the ocean taken as a whole over the Earth's history.

The way you manipulated your carbonate systems deviates from common approaches applied in coccolithophore physiology, where O(cean) A(cidification) effects are often investigated (thus, pH values are altered in these approaches – which is an important difference to your

data set). Similar to your approach: data at pH 8.3 of Bach et al. [9]. It is in my opinion important to note differences between these deviating approaches.

Authors's response: We did not aim to contribute to the OA research effort here, although the data can be subsequently explore in this way. As done in the work by Rickaby et al. (2010 Clim. Past), pH was kept constant to avoid a superposition of effects in our study. We only aimed to determine how coccolith stable isotope compositions evolve with higher carbon availability for the cells with a geological perspective.

l. 24: Introduction of "vital effect". Since the term is not uniformly used in literature (and not (yet :-)?) common in cocco community), I think a more precise definition of the term "vital effect" (I mean how you will refer to it throughout the MS, cf. eqn. (3)) would be beneficial for the reader.

Authors's response: This has been done, and was indeed useful for clarity. "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."

On this note, we have also homogenised the use of the vital effect for the carbon isotopes ($\delta^{13}C_c - \delta^{13}C_{DIC}$ vs. $\delta^{13}C_c - \delta^{13}C_{inorg}$ throughout the ms).

p. 15838
l. 11f.: You should clearly state here that the pH was set to a fixed value of 8.2. "A wide range of pCO2" could otherwise be misunderstood.

Authors's response: Done.

"As varying the availability of ambient DIC (primarily CO2)": As mentioned in a previous review for you (and also mentioned by referee 1), you need to clearly state that the prime carbon source of calcite is external HCO3− (you can see that in your data)!!! The carbon source of photosynthesis can be CO2 and/or HCO3− (in E. hux)!!! This is very important!!! This is for instance (indirectly though) shown in the study of Sekino and Shiraiwa [3] who you cite in line 15 (but see also (for instance): Paasche [10], Burkhardt et al. [11], Sikes et al. [12], and isotopic data in Rost et al. [13] and here in your study). CO2 limitation (in E. hux) occurs at CO2 concentrations below 10 µmol·L−1 (cf. e.g. Bach et al. [9]).

Authors's response: "primarily CO2" has been removed, also as per Referee#1 advice. In the revised ms, we do not favour one source over the other, as we do not want to enter into this controversy, and our dataset cannot tackle it.

You should compare your carbonate system ([CO2], [HCO_3^-], DIC, and total alkalinity (TA)) to ancient conditions here.

Authors's response: Same response as above. This is not feasible without making huge assumptions unfortunately.

p. 15840
l. 1f.: Why do you first remove all DIC from the water? You measured (and calculated) δ13C...

Authors's response: This method allows us to attain our targeted DIC more accurately, and more importantly to obtain the same $\delta^{13}C_{DIC}$ composition in all bioassays.

How much (mol·L−1) Cl− (and Na+) did you add? You should probably mention the increase (from ... to ...) in [Cl−]?

Authors's response: We see no change in TA before and after treatment. Changes in Na^+ and Cl^- are very minor compared to the original seawater concentrations (33 psu).

You should really add a table with the carbonate systems (incl. TA, DIC, CO2, HCO3−, CO32−, and maybe [Ca2+]) and also mention salinity. If you do not want to put it into the main document, you should definitely provide it as appendix.

Authors's response: Done.

l. 19f.: You should mention the cell densities. It is really a pitty that you could not measure PIC and POC per cell. Why did you not sample at higher cell densities then? This is probably how it is usually done, I suppose. As far as I know (I am not an experimentalist), the cell densities up to which you can sample (without signigicant carbonate system shifts) are ± known.

Authors's response: Growing more cells in each flask, especially for the low DIC levels, would have resulted in a shift in the composition and pH of the medium that may have altered the validity of our experiments.

Since Bach et al. (2013) used a similar carbonate system (the one at pH 8.3) at the same irradiance level (though different diurnal cycles), you may use their results to estimate the behaviour of daily POC and PIC production rates (e.g. POC/cell · dision rate), even though the strain deviates from the one you used...

Authors's response: Thanks. We have added this information in place of the reference to the work by Langer et al. (2006). "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."

At which time point (after onset of light) did you take samples to measure cell densities and cell sizes ...?

Authors's response: Approximately 3 hours after the beginning of the photoperiod (added in text).

p. 15841
l. 1ff: calculated specific growth rates should be listed in a table. How many replicates do you have?

Authors's response: Done. We did two replicates for each species and each DIC level (information added in text).

p. 15842

eqn. (2): How reliable are the calculations of δ18Oinorg and δ13Cinorg? It would make more sense to me to calculate " values that relate the calculated isotopic calcite signal to the isotope value of the external carbon source (i.e. relationship between δ13CC and δ13C HCO_3^- in terms of calcite, " is close to 0 (Rost et al. (2002)) ! almost no fractionation between ext. HCO_3^- and calcite ! HCO_3^- seems to be the carbon source of calcite ...).

Authors's response: The use of these inorganic references present the massive advantage to be independent of temperature, and hence eases comparison with other works.

We reiterate here that we only measure apparent fractionation in coccolith calcite. That coccolith $\delta^{13}C_c$ resembles values of bicarbonate ions does not represent a formal proof for the unique use of this DIC substrate from an isotopic point of view. But, once again, we do not want to revisit this controversy here or in our manuscript.

Well, in fig. 2 you give the d between δ13CC and δ13CDIC...

Authors's response: As explained above, we stick to an empirical calibration and conform to previous studies (as for the "$\delta^{18}O_c - \delta^{18}O_w$").

I don't understand why you give the difference between δ13CC and δ13CDIC (which is close to δ13C HCO_3^-) and not the difference between δ13CC and δ13CCO2 , when you assume that CO2 is the external carbon source. When plotting the latter value, you could see that (δ13CC - δ13CCO2) becomes much higher, i.e. the offset to zero increases strongly (δ13CCO2 d δ13C HCO_3^-)! This shows that HCO_3^- is the prime carbon source of calcite, not CO2.

Authors's response: See responses above (and also the first made to this Referee).

Calculating the ε value for oxygen would give evidence about how well the oxygen signal is correlated with the carbon source. Oxygen isotope effects are (even) more complicated than carbon isotope effects, because of their more complex interactions with other oxygen containing molecules, such as the ubiquitous H2O molecule.

Authors's response: $\delta^{18}O_c - \delta^{18}O_w$ values and $\varepsilon^{18}_{c\text{-}w}$ $\varepsilon^{13}_{c\text{-}DIC}$ values (now added in Table) are linearly correlated, hence we do not loose any information by adopting the well established δ – δ notation applied in foraminifera and coccolith researches.

p. 15842
l. 15f.: I miss the table with the corresponding values :-).

Authors's response: Done.

p. 15844
l. 17: why should growth rates (in the cited paper's title "metabolic rates") and cell sizes covary? "Metabolic rates" should be ± proportional to cell volume or POC/cell. Same for humans: 100 kg people exhibit higher respiration rates per individuum than 40 kg people... However, in respect to growth rates and cell sizes, I would have expected a negative correlation under nutrient limitation and maybe high light intensities where maximum speed of cell cycle cannot be increased any further... But that is another story. I should simply read the cited paper.

Authors's response: We are not sure we follow the argument made by the Referee here. This is indeed an excellent contribution to the field.

p. 15845
l. 5f.: Either, I misunderstand something, or: The regression lines are linear (Fig. 2 by the way), I cannot see an approximation towards the inorganic value. At 12mmol DIC·L−1, the value of E. hux is below the inorganic one.

Authors's response: Fig. Number corrected. *E. huxleyi* $\delta^{13}C_c$ values decrease with more external DIC. Indeed, at 12 mM, the values are slightly below inorganic, but only by 0.2 – 0.3 ‰. Then we feel that we can maintain our statement. These are biological data, and a spread exists between replicates.

p. 15846
DCUt as index for "internal carbon pool": Who's interpretation is that? CO2 cannot be the only carbon source of the cell! cf. e.g. your own data set. ...

Authors's response: Sentence (and concept) **removed**.

- Your interpretation of DCUt is strange, in my opinion.

Authors's response: See response above, in our previous ACs, and at the beginning of this letter.

- My interpretation of DCUt is that it indicates how much CO2 may be used for photosynthetic carbon fixation. The remaining carbon demand (of photosynthesis) would have to be covered by HCO_3^-. "μ · volume" gives an indicator for the photosynthetic (!!) carbon demand of the cell.

Authors's response: We agree with this statement, as it was clearly expressed in our original submission (reflecting the whole internal pool dynamics, and not specific to calcification).

"Growth rate (μ), cell size and ambient DIC (or CO_2) concentrations can be combined to generate an index accounting for the supply and photosynthetic utilisation of carbon by the cells (Rau et al., 1996; Bidigare et al., 1997; Burkhardt et al., 1999; Laws et al., 2002)." Sentence **removed**.

"We emphasise that this index is not specific to calcification, but rather, gives an estimates of the dynamics of the whole carbon inventory (pool), regardless its subsequent partitioning into photosynthetic ($CO_{2\ aq}$) or calcification (HCO_3^-) pathways." Anyway: Sentence **removed**.

"[CO2] · surface-area", in contrast, is used as indicator for the potential to take up CO2 via the surface-area (diffusive CO2 uptake is dependent on external CO2 concentration and the surface-area). Depending on which carbon species is used for photosynthesis, external CO2 or HCO3−, the internal isotopic carbon signal changes. As far as I know, Rau et al. and Bidigare et al. both worked with the isotopic carbon data of the organic phase (not calcite!). However, I do not doubt at all that the isotopic signal in calcite is influenced by photosynthetic activity.

Authors's response: We feel that we have already answered this above, and changed the ms accordingly, but we agree with the Referee's reasoning.

I think that μ · volume · surface area−1 does not change much in comparison to [CO2]. When altering light or nutrient conditions this may be different though. However, you should test the significance of the correlation between d and DCUt cs. d and [CO2].

Authors's response: We now clearly state that the primary driver is [DIC], division rates and cell geometry, themselves influenced by DIC have only a secondary role, as now explained in the discussion.

l. 10 CO2 is not the carbon source of calcite E. hux is not the only alga that can use CO2 as well as HCO3− for photosynthesis. This is a common feature, although it may be the only coccolithophore species for which a shift towards CO2 usage at high [CO2] has been measured.

Authors's response: We would have welcomed some references supporting this statement for other coccolithophores apart from *E. huxleyi* as to our knowledge there is almost no data on this point for species other than *E. huxleyi*. Regardless, this paragraph has been removed and we do not suggest that the *Ci* comes from ambient $CO_{2\ aq}$ assimilation by the cell anymore.

l. 25: Why should cells take up more HCO3− at high [CO2]? I don't understand this. You should also have in mind that you increased the concentration of HCO_3^- by a factor of 6, not only CO2. Kottmeier et al., in contrast, rarely changed HCO_3^-.

Authors's response: This paragraph was only an attempt to compare the DIC fluxes of the work by Kottmeier et al. (2014) with our isotopic measurements on coccolith calcite – this has been **removed**.

"carbon pool": this is in my opinion a "black box word". What is your (precise) interpretation of it? Do you simply mean the sum of all carbon species within the cell comprising all cellular compartments? Or is it rather a pool in the cytosol with locally enriched DIC, where the carbonate system is in chemical equilibrium? Why should a cell have such a pool? It is expensive to maintain, because the import of DIC (HCO_3^- ?) into this pool would have to function against a strong concentrational gradient (+ CO2 may leak out) and the import rates of DIC into the cell would have to be the same rates as without such a pool (because C fixation rate (C sink term) = C uptake rate (C source term), if DIC pool remains constant over time) .... i.e. high C fixation rates = high C uptake rates and vice versa [cf. 14]. Or may the pool even comprise organic components? However, the type of "pool" would have a major impact on the isotopic
signal ...

Authors's response: We now clearly indicate: "**inventory** of internal DIC species" in the text to explain this idea of a carbon pool (see e.g. Nimer et al., 1992 – ref now added in the ms). We are not sure about the arguments presented here. To achieve the photosynthesis and calcification rates under ambient carbon conditions, coccolithophores *must* have an intracellular reservoir of carbon. In some cases, this pool can be measured using silicone oil centrifugation, or ^{14}C pulse chase experiments which probe the acid labile carbon contents of the cells or the "internal pool" see Isensee et al., 2014; Sekino and Shiraiwa, 2008, Nimer et al., 1992.

p. 15847
l. 1/2: Where are the data that tell you that? Do you think C limited cells store C internally? Why should they do such a thing?

Authors's response: This feature is evidenced by the overall fertilising conditions induced by the addition of DIC around the cells, with increased growth rate from 2 to 4 mM.

DIC is the sum of CO2, HCO3− , and CO32−(+ H2CO3) and I suppose the cell reacts to all C species differently, depending on the type of import mechanism for instance.

Authors's response: Yes.

l. 12: reference to Fig.

Authors's response: Done.

secs. 3.4.1 and 3.4.2: As mentioned above already, I think the correlations may be better with CO2 than with DCUt.

Authors's response: See Interactive Discussion and responses above. We do not refer to this index anymore.

p. 15848
sec. 4.1: How do you explain that δ13C and δ18O both increase or both descrease for one species? Isn't that counter-intuitive when considering CO2 in the external medium equilibrium) being depleted in 13C, but enriched in 18O compared to HCO−
3 ?

Authors's response: This is the point of section 4.2.

p. 15849
l. 2/3: Inorganic or organic precipitation? Did Anning et al. do precipitation experiments? Watkins and Hermoso: O isotopes, not C isotopes?

Authors's response: Inorganic. The references Anning et al., 1996; Watkins et al., 2014; Hermoso, 2015 have been deleted.

l. 5: "co-evolution of DIC": I don't understand that. A reference is missing

Authors's response: This sentence now reads: "Thermodynamically, the mechanisms and the dynamics of $^{18}O/^{16}O$ fractionation are very different to those described for the carbon isotopes (Zeebe and Wolf-Gladrow, 2001)".

l. 22: No. We did not separate photosynthesis and calcite precipitation for DIC sourcing in our early coccolithophore model Holtz et al. [8]. As a result, we find that CO2 and HCO_3^- interconversion inside the cytosol is low, leading to a separation between CO2 and HCO_3^- fluxes through the cytosol. In a refined model version [15], we even explain the observed

increase in PIC production rates at low [CO2] with the elevated uptake of HCO_3^- for photosynthesis – which strongly opposes the idea of separated DIC sources for photosynthesis and calcite precipitation!

Authors's response: Noted. The corresponding paragraph has been **removed**.

Bach et al. (2015) did not intend to describe carbon fluxes mechanistically. They used correlations between seawater chemistry and POC and PIC production for their model. This model is neither based on internal pools nor on a mechanistic explanation of cellular carbon fluxes.

Authors's response: Same here.

l. 23-29: HCO_3^- is the prime external C source of calcite (cf. references I listed before). There is no well-grounded experimental evidence (known to me) arguing against this (at least not for E. hux – and you just presented evidence for HCO_3^- usage for three more cocco species in your data set). In case you know something else please let me know. This (HCO_3^- as carbon source for calcite) does, however, not imply that the isotopic carbon signal of calcite is not altered by photosynthetic activity...

Authors's response: As pointed by the Referee, only a comprehensive model requiring coeval $\delta^{13}C_{org}$ values and PIC/POC ratios could formalise this HCO_3^- / $CO_{2\,aq}$ "controversy". These lines have been **removed**.

p. 15850
l. 1-5: The RubisCO effect (Spero et al. 1997) is one more argument for the potential of an isotopic signal to spread from the symbionts through the host and into the calcifying space (cf. model of Zeebe et al. (1999))

Authors's response: We agree with this statement but we are unsure how (*if?*) this information ought to be mentioned in our ms.

l. 2: To me, "12C depletion" sounds as if [13C]/[12C] > 1

Authors's response: This has been reformulated using inorganic calcite as a reference.

l. 13: Langer et al. (2009) used carbonate systems with DIC d 2mM and changing pH values. You may rather use the data set of Bach et al. (2013) (cf. above) who used a similar carbonate system set up as you (the one at pH 8.3). PIC/POC increases with increasing DIC due to a decrease in POC.

Authors's response: This is true. The work by Bach is more suitable for comparison with our own study. We have removed here the reference to the paper by Langer, and replaced it with reference to the bach's paper with the observation of the data made by the Referee.

l. 16: wrong figure reference? By the way, units in figures 1 and 2 are not correct: mM/kg. I guess you mean mmol/kg or mmol/L? Previously you used mM, so mmol/L ... it may be good to stick to one unit?

Authors's response: Reference to Figure has been corrected and units corrected. Thanks for spotting this.

l. 16: what is a lower degree of carbon utilisation? Do you mean lower carbon fixation? Or DCUt?

Authors's response: We mean a relatively lower utilisation of the internal DIC compared to the supply (hence a lower DCUt when we were referring to this index in the original version of the ms).

It would be so much easier if you would give your data in ε values and would have defined "internal carbon pool" precisely...

Authors's response: One again, we conform here to palaeoceanographic usage. All previously reported culture fractionation coefficients have been given via the "δ – δ" notation (coccoliths and beyond). We have nevertheless added the corresponding ε values in the table. By internal carbon pool, we mean the inventory of DIC in the cell (see above - now specified in text).

I miss the statement that CO2 is isotopically depleted in 13C compared to HCO_3^-. My interpretation of all data HCO_3^- is the prime carbon source of calcite. For E. hux and P. placo data CO2 diffuses across membranes. Thus, at high DIC, where [CO2] is very high also, CO2 enters the cells and brings in a lot of 12C (comp. to 13C). Most organic carbon might be built from CO2 at these conditions. But this is unfortunately not measured. Would have been very interesting to see the corresponding values for the organic phase ... For the other two species, this effect seems to be superimposed by other effects. Cellular structure, PIC and POC production rates, as well as the isotopic signal in biomass may give further evidence ... Concerning the data of leptoporus and pelagicus: are MIMS etc. data available here? What do they say?

Authors's response: To our best knowledge, there is no MIMS data available for *C. leptoporus*. We wonder why this ^{12}C-rich influx would be restricted to *E. huxleyi* and *P. placolithoides* since CO_2 can diffuse across all membranes. This explanation therefore does not reconcile the interspecies response. Instead, and as pointed by the same Referee in her review quoting the data by Bach et al. (2013), reduced POC production may better account for decreased $\delta^{13}C$ values in these two species, as now explained in text.

p. 15851
l. 3: H+ hypothesis: would you not first expect a reduction in the precipitation rate? ok, you don't have PIC production data ...

Authors's response: We are indeed unable to answer this.

l. 13: The data of Langer et al. (2006) are not comparable to your data set. DIC varies around 2mmol·L−1, pH is lower at high [CO2] which is thought to reduce PIC, I suppose (I right now did not have another look at the data)... thus, a completely different set-up

Authors's response: We have removed the reference to the work by Langer.

l. 22: Changes of δ18O (changes in which which direction?) may originate from increased proportion of HCO_3^- over ....

Authors's response: Sentence deleted.

sec. 4.2.2: This section is even more difficult to follow than previous deiscussion.

Authors's response: We have shortened (as per Referee#3's suggestion) and reformulated this paragraph, which is now easier to read (see changes track version attached).

Do you have any experimental evidence (apart from your interpretation of the carbon isotopic signals which should in my opinion rather be discussed in terms of the carbon source) for an increase/decrease in the residence time/overturning rate? Values such as PIC or POC production (C fixation) rates versus C uptake rates? Nevertheless, I do not doubt that residence time (separation between 12C and 13C necesary) can alter the isotopic signal...

Authors's response: We were trying to parallel the approach used by Bidigare et al. (1997) and many others, and as stated in text, to generate a view of the degree of utilization of an internal pool and how it matched with the degree of vital effect expressed, so using the index $\mu \times$ volume / $Ce \times$ surface-area to approach a carbon supply-to-demand balance by the cells (see Hermoso, 2015 – Paleoceanography). However this is now **removed** from the manuscript.

You seem to discuss different carbon sources here. I am wondering why you did not discuss this issue for carbon isotopes which would be more obvious to me than with oxygen isotopes, whose reactions comprise much more reactions than the carbonate system ... but maybe I got you wrong here.

Authors's response: This section has been shortened as also recommended by Referee#3. We do not discuss potential sources used for calcification in the revised ms. As pointed by Referee#2 earlier, carbon and oxygen isotopic systems are way different so that it is difficult to draw a parallel discussion on the two elements (time dependent isotopic exchange between DIC and H_2O for O does not apply on C). Furthermore, existing literature on coccolith (and foraminifera) show a definite focus on oxygen isotopes, hence there are more hypotheses open for discussion. This is due to the widely used $\delta^{18}O$ temperature proxy.

I stop here, since I have too many open questions ...
I hope my comments are helpful for you,
Lena Holtz

Technical comments

p. 15840
l. 9: To obtain

Authors's response: Done.

l. 10 mMk−1
sw?

Authors's response: mmol kg_{sw}^{-1}.

p. 15841
15-19: Which standard did you use for dO? L. 15: V-PDB; l. 19: V-SMOW –
I am confused. Cf. next page, l. 6-8.

Authors's response: The same standard (derived from the V-PDB) is used for both carbon and oxygen isotope ratios. A different thing is the scale: oxygen isotope of calcite is expressed in V-PDB, whereas seawater composition is expressed against V-SMOW.

l. 25: Here, you should first introduce the calculations of δ13CC and d8OC. –
where are the values?

Authors's response: We feel that this is common knowledge and would disrupt the flow of the Methods section.

l. 26: "offset of coccolith calcite from inorganic" (add) calcite.

Authors's response: Done.

p. 15842
l. 19: "per day (μ d 0.7 d−1)": Not everyone is familiar with the difference between specific growth rate and division rate. You should hence say that this μ value corresponds to 1 divion per day. E. hux actually reaches values μ > 1.

Authors's response: A value of μ of 0.7 corresponds to one division per day (population doubling everyday).

l. 21: for reasons of comparability, you should mention cell diameter/radius of E. hux also.

Authors's response: Done.

p. 15843
l. 1: δ13C = δ13CC?

Authors's response: Yes, a "$_c$" has been added.

l. 2: 280 is the pre-industrial value :-)

Authors's response: "(pre-industrial)" has been added.

l. 2: where is this shown? Reference is missing. Table? Figure?

Authors's response: We have added reference to Fig. 2a here.

l. 1-3: do you speak about the vital effect or δ13CC?

Authors's response: This has been clarified: "(hence, a "positive" ^{13}C vital effect)".

l. 3: "very large": is this very large?

Authors's response: We regard an interspecies offset of 3 ‰ as very large indeed.

l. 6: You cite Fig. 2 before Fig. 1. Description in text and figure do not belong together.

Authors's response: This has been corrected.

l. 12f.: what do you mean with "cultures were implemented"? inoculated? Grown? Kept at? Or did you implement a model?

Authors's response: We have changed "implemented" by "grown" to avoid confusion.

p. 15844
l. 1: it can be, not been

Authors's response: Changed.

3.2 "Effect of increased DIC" (at constant pH) " on growth .... "

Authors's response: "at constant pH" has been added.

l. 17-18: is this a sentence?

Authors's response: This long sentence has been divided into two shorter ones for clarity and now reads: "Overall, there is no covariation between growth rates and coccosphere and cell sizes for the species examined here (Fig. 1a; Fig. 1b). One may expect decreased μ to be accompanied by longer generation time, and hence larger cell sizes (Aloisi, 2015). Nevertheless, the data indicate 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)".

p. 15844
l. 20: "become relatively larger"

Authors's response: We have changed "species" for "cells".

p. 15845
l. 3: wrong Fig. cited?

Authors's response: Corrected.

l. 19: vital(skip s) effect: is defined differently in brackets here and eqns. (2) and (3) ...

Authors's response: Done.

p. 15848
l. 19: "such contrasting responses": reference to which responses?

Authors's response: We have added "interspecific" response.

p. 15850

l. 1: “Specific to photosynthetic”: I do not understand what you mean here.

Authors’s response: The sentence now reads: “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 *Ci* leading to substantial isotopic consequences on the stable isotope composition of biominerals (McConnaughey, 1989; Spero et al., 1997; Hermoso et al., 2014)”.

l. 1/2: The sentence is weird.

Authors’s response: See previous answer.

l. 2: “coccoliths” should read coccolithophores

Authors’s response: Done.

l. 3: you should introduce RubisCO here (I mean the full name)

Authors’s response: Done.Although it should actually be RuBisCO

Referee#3 (Anonymous)

The work by Hermoso et al. is a valuable contribution to the complex field of carbon and oxygen isotope fractionation in coccolithophores. The multi-species approach is particularly helpful and besides providing much needed insights, raises, as is so often the case, many additional questions. This is not a bad thing, of course, and I appreciate the author’s attempt to tackle some of them in considerable detail.

Authors’s response: We are grateful to this Referee for acknowledging the relevance of our work, and pinpointing the challenge to undertake a biogeochemical study for palaeoceanographic purposes, as the concepts are really different leading to misunderstanding between the two worlds.

However, I think that sections 4.2.1 and 4.2.2, although a nice exercise, bring little to the table in terms of the central section on proxy development (4.3). Moreover, section 4.2.1 for instance uses the PIC/POC ratio as one important parameter in the argument. But in the present study PIC/POC was not determined. So I feel that such highly conjectural sections (4.2.1 and 4.2.2) take too much space and could be shortened considerably.

Authors’s response: Some sentences of these two sections have been shortened to avoid vagueness, in particular we removed two paragraphs from the oxygen isotope section. However that the elements of discussion presented here are necessary to bridge biogeochemical concepts to palaeoceanographical implications (indeed presented in section 4.3). Thanks to Referee#2, a more suitable study allows more meaningful comparison of data (PIC/POC) published literature data (Bach et al., 2013) for *E. huxleyi*.

Concerning DCUt, I would suggest keeping it as it is used in section 4.3.

Authors's response: As discussed with Referee#2 during the discussion phase, as following the AE's guidance, we have removed the DCUt index. We will show the correlation with external total DIC in the ms.

Thanks to the thorough comments by the other reviewers there is not much left to say, from my point of view. I will merely highlight a few technical points which might even have been mentioned by the others (if so sorry for that).

1 Please state explicitly which parameters of the carbonate system were used to calculate it, and how it was calculated.

Authors's response: Done (in new Table 1).

2 Give the full carbonate chemistry in a table (and preferably the other data as well)

Authors's response: A Table with all numerical data has been prepared.

3 How many replicates were run?

Authors's response: Two replicates for each species and each DIC conditions were done. This is now stated in the body text.

4 It would be helpful to have a figure showing cell density on y and time on x as an illustration of the semi-continuous approach

Authors's response: Unfortunately, we are unable to produce such graphs with the data we have.

5 In section 3.3 you say that you used coccosphere size as opposed to (naked) cell size. Actually, your Coulter Counter measurements are probably much closer to naked cell size than to coccosphere size. This type of machines is hardly capable of "seeing" the coccosphere.

Authors's response: In other studies, we obtained two distinct peaks on the Counter Counter by measuring coccospheres and decalcified cells. The difference for each species is above the sensitivity of the instrument, and the standard deviation of the size distribution of the latex beads (5 and 10 microns in diameters) used to calibrate the Coulter Counter. Therefore, we prefer indicating coccosphere sizes rather than cell sizes, as we effectively measured untreated (unacidified) coccolithophores.

Changes tracked version

Vanishing coccolith vital effects with alleviated carbon ~~CO_2~~ 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 ~~lacked any~~ isotopic offsets from inorganic calcite compared to the substantial vital effects expressed at ~~whereas in~~ low (present-day) DIC concentrations~~, these vital effects and interspecies differences become substantial~~. ~~The present study. From a mechanistic viewpoint, we show that the vital effect .~~ 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. ~~and a modulation of these parameters with ambient carbon availability~~. 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 ~~on coccolith isotopic compositions~~ by demonstrating ~~the threshold for the absence of vital effects under high DIC regimes~~ 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 ~~of~~ 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 ~~($\delta^{13}C$, $\delta^{18}O$, $\delta^{11}B$, Δ_{47})~~ 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. ~~as in~~ 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 the present-day 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 constant pH (8.2) recreated in the laboratory. As varying the availability of ambient DIC ~~(primarily CO_2)~~ substrate may modulate the degree of carbon limitation for algal growth (cell division rate and size) ~~and influence the dynamics of the internal carbon pool (Sekino and Shiraiwa, 1994; Laws et al., 2002; Rickaby et al., 2010; Aloisi, 2015; Hermoso, 2015)~~, this culture approach will allow us to determine ~~the static *versus* dynamic nature of the vital effect in coccolith calcite in response to CO_2~~ 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 ~~with a geological perspective~~.

2 Material and methods

2.1 Coccolithophore strains studied

Emiliania huxleyi has attracted most recent attention ~~on~~ in coccolithophore research due to its dominance in present-day oceans, ~~and the consecutive~~ its importance in biogeochemical cycles, and accompanying relevance to ~~study it with ongoing concerns about our Anthropocene~~ 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 ~~would only concern~~ a narrow time interval as this species has only recently evolved (~ 268 kyr ago; Thierstein et al., 1977). ~~Yet, coccoliths of this species represent a suitable material for Pleistocene studies with further insights to our Anthropocene oceans.~~

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

psu) 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 obtained the desired DIC level (2; 4; 6; 8; 10 and 12 mmol kg_{sw}^{-1}), 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 to generateing 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:

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

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).

$$^{18}O \text{ Vital effect} = \delta^{18}O_c - \delta^{18}O_{inorg} \quad (2)$$

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) ~~for~~ 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.

$$^{13}C \text{ Vital effect} = \delta^{13}C_c - \delta^{13}C_{inorg} \quad (3)$$

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 DIC)

3.1.1 Growth rates and coccosphere sizes

Cell division rates at present-day (pre-industrial) DIC levels (~ 2 mmol) 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 ($\mu \sim 0.7$ 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 ~~size~~ diameter between *E. huxleyi* and *P. placolithoides* (~ 10 µm) exhibits the lowest division rates among all examined species at 2 mmol 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 present-day CO_2 levels (~ 270 ppm / ~ 2 mmol DIC) is considerable, on the order of 3 ‰ (Fig. 2a). This variation ~~is due to~~ 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 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 range. *C. pelagicus* and *C. leptoporus* meanwhile have relatively similar $\delta^{13}C_c - \delta^{13}C_{~~DIC~~inorg}$ values, corresponding to a ~~of~~ −2.5 ‰ vital effect ~~below that of inorganic calcite~~. These numbers are in good agreement with published literature when cultures were ~~implemented~~ grown at low cell concentration (see synthesis in Hermoso, 2014).

3.1.3 Oxygen isotope composition of coccolith calcite

The $\delta^{18}O$ of coccolith calcite grown by algae exposed to 2 mmol 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 heavy $\delta^{18}O$ ratios ~~(calculated using the equation by Kim and O'Neil, 1997)~~. *Calcidiscus leptoporus* ("light group") exhibits lighter $\delta^{18}O_c$ 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~~en~~ 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 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, 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 range of DIC concentration, but decreased growth rates are ~~drastic~~ 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*. Overall, there is no covariation between growth rates and coccosphere and cell sizes for the species examined here (Fig. 1a; Fig. 1b). ~~as o~~One may expect decreased μ to be accompanied by longer generation time, and hence larger cell sizes (Aloisi, 2015).

Nevertheless, the data ~~indicate~~ confirm that both *E. huxleyi* and *P. placolithoides* ~~species~~ 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 ~~response~~ change in size with DIC availabity ~~for this parameter~~, 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 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 ~~becomes~~ indistinguishable from ~~to~~ 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 (*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 datapoints departs from the 4 – 12 mmol linear trend with substantial low $\delta^{13}C$ values. This "jump" in *C. pelagicus* $\delta^{13}C$ values between 2 and 4 mmol 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.

~~Overall, a noteworthy feature of the data is that all the vitals effects ($\delta^{13}C_c - \delta^{13}C_{DICinorg}$ values) converge within a narrow range comprised between + 0.5 and +1 %0 to – 0.5 ‰, representing an almost complete erasing of the original (measured under 2mmol of DIC) interspecific differences in carbon isotopes.~~

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 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 of DIC, yet representing a substantial decrease in the magnitude of the vital effect compared to the 2 mmol measurement. The large species *C. pelagicus*, assigned to ~~the isotopic~~ a near-inorganic (sensu Kim and O'Neil, 1997) ~~equilibrium~~ group shows ~~a~~ constant~~,~~ ~~vital effect-free~~ $\delta^{18}O$ values with a limited vital effect, regardless of changes in ambient DIC concentrations.

~~3.3 Estimates of the degree of utilisation of the carbon internal pool~~

~~Growth rate (μ), cell size and ambient DIC (or CO_2) concentrations can be combined to generate an index accounting for the supply and utilisation of carbon by the cells (Rau et al., 1996; Bidigare et al., 1997; Burkhardt et al., 1999; Laws et al., 2002). This widely used metric for the degree of utilisation of the internal carbon pool relies on the assumption of molecular CO_2 passive diffusion in phytoplankton (Bidigare et al., 1997; Laws et al., 2002; Hermoso, 2015). Assuming that the passive influx of CO_2 constitutes the only source of carbon to the cell, the flux of carbon is proportional to $[CO_{2\,aq}]$ × surface area, and carbon usage is proportional to μ × volume (assuming constant carbon density). The ratio of carbon supply to carbon usage (Eq. 4) reflects the index describing the degree of carbon utilisation, hereafter referred to as "*DCUt*". As the increase in ambient $[CO_2]$ concentration (from low to high DIC concentrations) was the same for all species, interspecific differences in this index were driven by growth rates and coccosphere size.~~

~~$$DCUt = (\mu \times \text{volume}) / ([CO_{2\,aq}] \times \text{surface area}) \quad (4)$$~~

~~In the present study, we used coccosphere size rather than naked cellular size to account for both organic and inorganic (calcite) carbon fixation. As coccosphere and cell size are linearly related (Henderiks, 2008), this slight adaptation of the original formula does not alter the validity of the calculated *DCUt* index. Furthermore, this proxy implicitly considers a carbon assimilation from aqueous CO_2. The species *E. huxleyi* is the only coccolithophore algae, which has been reported with the ability to shift from CO_2 to HCO_3^- assimilation under ambient carbon limitation (Kottmeier et al., 2014; Bach et al. 2014). We are aware of no evidence for increased HCO_3^- assimilation in coccolithophores under high CO_2 environments,~~

so we assume prominent CO_2 influx at high DIC, consistent with the work of Kottmeier et al. (2014) and Hermoso (2015).

For all species, increasing DIC led to less utilisation of the carbon pool explained by alleviated carbon limitation, although the relative changes differ among species. This effect is considerable for the largest cell, namely *C. pelagicus*, especially apparent between 2 and 4 mM of DIC. At maximum DIC concentration, all species converge to similar ($[CO_{2\,aq}] \times$ surface-area) / ($\mu \times$ volume) values, possibly indicating that carbon replete internal conditions are attained, except for the coastal species *P. placolithoides* that has a significantly higher *DCUt* index at 12 mM compared to all other species (Fig. 1c).

3.4 Link between the magnitude of the vital effect and the degree of utilisation of the carbon internal pool

3.4.1 Coccolith carbon isotope composition

In *C. pelagicus*, there is a large relative change in the *DCUt* index and $\delta^{13}C_e$. These two parameters are statistically linked with a linear relationship ($r^2 = 0.98$): the greater *DCUt*, the more negative $\delta^{13}C_e$. The "jump" in carbon isotope composition of *C. pelagicus* calcite also corresponds to a large difference in the *DCUt* between 2 and 4 mM DIC.

In other species, there is a narrower spread of $\delta^{13}C_e$ and *DCUt* values, and coccolithophore algae show contrasting relationships between these parameters. *Emiliania huxleyi* and *Pleurochrysis placolithoides* exhibit decreased $\delta^{13}C$ values with a lower degree of internal carbon utilisation with linear fits ($r^2 = 0.82$ and 0.60, respectively). It is worth noting that the non-linear nature of the statistical link mostly arises at the lowest DIC (2 mM) concentrations. In *C. leptoporus*, the response is somewhat comparable to that described for *C. pelagicus* with the exception of the 2 mM datapoints which are isotopically heavier than predicted by a linear fit for this species.

3.4.2 Coccolith oxygen isotope composition

The observed changes in coccolith $\delta^{18}O$ are correlated with the *DCUt* index with the notable exception of *C. pelagicus*. For this large species, near-equilibrium oxygen isotope composition is always expressed with no effect of $[CO_{2\,aq}]$, [DIC] or coccosphere size. This result differs from other examined species. In *E. huxleyi* a linear fit ($r^2 = 0.68$) links the

~~decreased DIC (hence, potentially under carbon limitation) and coccolith δ^{18}O, leading to a smaller expression of the ^{18}O vital effect. Species from the light group show similar behaviour with δ^{18}O tending towards equilibrium with alleviated carbon limitation (at high DIC), albeit with linear trends (r^2 = 0.77 for *C. leptoporus* and 0.76 for *P. placolithoides*).~~

~~Finally, we observe that the data for *C. pelagicus* in both carbon and oxygen isotope systems are compatible with those reported by Rickaby et al. (2010), but in the present study, we extend the isotopic response of these species to a higher range of DIC, from 8 to 12 mM (equivalent to a range of 1400 - 2200 pCO_2 level).~~

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, δ^{13}C and δ^{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 δ^{13}C and δ^{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 δ^{13}C composition is insensitive to temperature, precipitation rates and geologically relevant seawater pH values (Romanek et al., 1992~~; Anning et al., 1996; Watkins et al., 2014; Hermoso, 2015~~). ~~In inorganic calcite, the co-evolution of [DIC] and the magnitude of~~ Thermodynamically, the mechanisms and the dynamics of ~~^{18}O/^{16}O~~ oxygen isotope fractionation are ~~is~~ very different to ~~that~~ those described 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~~; Watkins et al., 2013, 2014~~). This effect can be as high as 1.5 ‰ for δ^{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. ~~Before new proxies are developed to overcome these biogeochemical uncertainties, the best quantitative approach for the supply and utilisation of carbon by the cells remains the *DCUt* index (Eq. 3).~~

~~The correlation between *DCUt* and coccolith isotopes shown in the present study ought to be further explored, particularly to establish a causal link. Originally established for quantifying the magnitude of carbon isotope fractionation in phytoplanktonic organic matter (Rau et al., 1996; Bidigare et al., 1997; Laws et al., 2002), this proxy has also proven relevant for coccolith calcite including their oxygen isotopes (Hermoso, 2015). It has to be pointed out that the parameter used to calculate the *DCUt* proxy is not specific to coccolith (calcite) formation, but rather reflects both photosynthetic and calcification carbon usage dynamics in the algae. Recently published modelled carbon fluxes in coccolithophores tend to separate photosynthesis and calcification for DIC sourcing (Holtz et al., 2015; Bach et al., 2015). We emphasise that our understanding of the internal carbon pool build-up favours a preponderant CO_2 assimilation by phytoplanktonic calcifiers and that both pathways use a common internal carbon pool (Sekino and Shiraiwa, 1994; Bolton and Stoll, 2013; Hermoso et al., 2014; Kottmeier et al., 2014). Hence, the assumption that calcification utilises bicarbonate ions transported from the extracellular environment to the coccolith vesicle with no influence from photosynthetic carbon fixation conflicts with many physiological and isotopic evidence.~~

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 ratios. 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 enhanced intracellular carbon dynamics to lowered vital effects~~

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

4.2.1 Carbon isotope system

~~Specific to~~ In photosynthetic, or photosynthetic-associated biomineralisers such as the foraminifera, corals and coccolithophores, ~~is the super-imposition of~~ 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 *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: $\varepsilon^{13}_{CO2\ aq - HCO3^-} \sim 10$ ‰ and $\varepsilon^{13}_{calcite-HCO3^-} \sim 1$ ‰ at 15 °C; Zeebe and Wolf-Gladrow, 2001). By contrast, ~~whereas~~ *C. pelagicus* and *C. leptoporus* have lighter carbon isotopic composition at ambient (2 mmol) DIC levels, falling between $\delta^{13}C$ of $CO_{2\ aq}$ and $\delta^{13}C$ of HCO_3^-, hence are lighter in carbon isotope composition than 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-~~depletion~~ fixation by photosynthesis ~~photosynthetic carbon fixation~~ (Laws et al., 2002; Benthien et al., 2007; Hermoso et al., 2014; Tchernov et al., 2014). ~~Langer et al. (2009) showed that the PIC/POC ratios in RCC 1256 *E. huxleyi* (the strain being examined here) were constant (~ 0.8) with changing carbonate chemistry, albeit on a narrower range of DIC than that of the present study.~~ 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, ~~to explain~~ the lowered *E. huxleyi* ~~coccolith~~ $\delta^{13}C$ measured at high DIC concentrations in the present study ~~with a lower degree of carbon utilisation (Fig. 1c)~~ are the likely consequence ~~we suggest that the isotopic composition~~ of the internal carbon pool becom~~es~~ing less imprinted by ^{12}C photosynthetic-driven Rayleigh fractionation because the latter process is "diluted" in a larger internal carbon pool. ~~Hence, a similar biogeochemical control operated exerted by the *DCUt* and the size of the carbon pool seems to set $\delta^{13}C$ values of both organic matter (see Bidigare et al., 1997) and coccolith calcite produced by *E. huxleyi* (data of the present study).~~ As aqueous CO_2 is isotopically lighter than HCO_3^- ions, an alternative mechanism relying on a shift from HCO_3^- to CO_2 cellular acquisition under elevated DIC concentrations can be hypothesised, as it would match the biological data for *E. huxleyi* reported by Kottmeier et al. (2014).

Species originally with very light $\delta^{13}C$ values at 2 mmol 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). ~~Adopting the inverse reasoning of that made for the ^{13}C "light heavy group" (*E. huxleyi*), it may be expected that for *C. pelagicus* and *C. leptoporus*, there is enhanced production of POC relative to PIC if the mere isotopic control on coccolith $\delta^{13}C$ was indeed photosynthetic $^{13}C/^{12}C$ Rayleigh fractionation.~~ 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). ~~One prominent feature is indeed that elevated ambient DIC concentrations result in decreased growth rates, but also in coccosphere, and cell size and PIC/POC ratios (Rickaby et al., 2010). These changes lead to a relatively large decrease in the *DCUt* index compared to other species, implying a relatively small internal carbon pool, and as a consequence, the expression of a large ^{13}C vital effect in coccolith calcite.~~ 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 heavier 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 ~~was a consequence of~~ is an isotopic relic of a partial ~~prominent~~ 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). ~~Changes in coccolith $\delta^{18}O$ and in the magnitude of ^{18}O vital effect may hence originate from an increased supply of proportion of HCO_3^- over CO_2 to the *Ci* (hence lowering coccolith $\delta^{18}O$) or arising from a longer residence time that will re-equilibrate ^{18}O excess of the DIC pool (leading to $\delta^{18}O$ closer to equilibrium conditions). Previous studies on coccolith biogeochemistry, and particularly those focussed on the oxygen system have revealed a control of the *DCUt*, and potentially on the overturning rate of the internal carbon pool (Hermoso, 2015). This relationship is also apparent in our dataset (Fig. 3b). Under carbon limited growth conditions (low *Ce*), there is may be a fast turnover of the~~

~~internal carbon pool.~~ 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* ($\varepsilon^{18}_{CO2\ aq-HCO3^-}$ = 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*. ~~that controls the record of isotopic disequilibrium of the DIC system due to incomplete re-equilibration of CO_2 with whole DIC and H_2O reservoir at the time of calcification in the coccolith vesicle (Hermoso et al., 2014). We note that unifying carbon and oxygen isotopes of coccolith calcite, the data do not support any shift from CO_2 to HCO_3^- assimilation by the cells.~~

In the present study, in all species except *C. pelagicus* that always displays near-inorganic $\delta^{18}O$ values, a ~~causal~~ link between ~~*DCUt* and,~~ [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. ~~This process may explain why $\delta^{18}O$ of *E. huxleyi* significantly decreases and converges towards the composition of inorganic calcite under higher [DIC].~~ Comparing our isotopic 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*, ~~the inferred~~ 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 ~~This lack~~ expression of very limited ~~of an~~ ^{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 ~~$^{18}O/^{16}O$~~ 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. ~~As to date we are still unable to identify the biogeochemical mechanisms leading to $\delta^{18}O$ being more negative than inorganic calcite for *C. leptoporus* (Candelier et al., 2013), insights into changing magnitude of the vital effect with more carbon availability for this species may help understanding "light group" dynamics. A way to address this question is a comparison between *Pleurochrysis* and *Calcidiscus* sp., both having relatively low $\delta^{18}O$ values. The most apparent commonality between these two species is relatively similar cell size. However, they are significantly different in terms of PIC/POC ratios, specific growth rates, coccolith size and $\delta^{13}C$ composition. One means to explain a low calcite $\delta^{18}O$ signature is by large kinetic effects including fast precipitation rate, potentially associated with a higher saturation state in the coccolith vesicle (Watkins et al. 2014). In the absence of physiological studies on *C. leptoporus*, or on its biomineralising toolbox, it is difficult to discuss this hypothesis further. Uniquely observed in the largest cells, *C. leptoporus* decreases its specific growth rate with higher DIC availability with relatively unchanged coccosphere diameter. This may lead to increased CO_2 influx to the cell, and as an isotopic consequence, intracellular oxygen isotope re-equilibration is longer and calcite gets~~

~~isotopically heavier and closer to inorganic conditions. Under this assumption, the original cause of the isotopic light group has to rely on a source of ^{18}O-rich DIC, possibly locally high [CO_3^{2-}] in the coccolith vesicle, but the origin of such a source remains elusive (Suffrian et al., 2011; Bach et al., 2013; Bach et al., 2015).~~

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 ~~absence~~ 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 lighter 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 ~~these~~ 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, 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. Overall, 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 pCO2 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.

~~The hypothesis by Bolton and Stoll (2013) about a possible "Late Miocene threshold" at about 575 – 375 ppm of atmospheric CO_2 (assumed to correspond to a range between 12 – 19 µM of aqueous 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). We observe, however, that through this range, other coccoliths show progressive isotopic trends (Fig. 2a). In our experiments, above a threshold of 10 mmol of DIC in the culture medium (corresponding to atmospheric composition of 1600 ppm of CO_2 – *the inferred concentrations that prevailed during the Palaeogene*), there is unsubstantial vital effect in coccolith oxygen isotopes.~~

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 $\delta^{18}O$) are they 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 ~~*DCUt* index derived from best correlated with size and growth rate, but also~~ contains an important environmental parameter sought in palaeoceanography, namely DIC concentrations. ~~As it appears that there is a strong coccosphere size component related to – *and possibly controlling* – the magnitude of the vital effect, especially for $\delta^{13}C$ values, a coccolith size-based proxy can be used in turn to derive palaeo-DIC concentration in the geological record.~~ 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 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. ~~A "reverse" approach using the present calibration utilising the magnitude of the vital effect, appears possible to derive *DCUt* estimates that can be, in turn, linked to [$CO_{2\,aq}$] concentrations.~~ 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 ~~*DCUt*~~ in the absence of preserved coccospheres in the sedimentary register, except in some peculiar settings (Gibbs et al., 2013).

5 Conclusions

This work provides ~~mechanistic~~ 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.

~~**Data availability**~~

~~The data used for the present study will be made available on the Oxford Research Archive (ORA-data) website (http://ora.ox.ac.uk).~~

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Figure captions

Figure 1. Changes in algae specific growth rates (panel a) and~~,~~ coccosphere diameter (panel b) ~~and the degree of utilisation of the internal carbon pool (panel c)~~ on a range of 2 to 12 mmol ~~M~~ of DIC per kg of seawater in the culture medium. ~~Equivalent aqueous CO_2 concentrations in each batch are given for reference at the bottom of panel c.~~ 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. Correspondance between DIC concentrations and pCO_2 levels were obtained via the CO2Calc software (Table 1).

~~**Figure 3.** Changes in coccolith carbon and oxygen isotopes with the degree of utilisation of the internal carbon pool (referred to as *DCUt* index in text – see Eq. 4). We observe relatively good relationship in the evolution of the vital effects via the degree of utilisation of the internal carbon pool in various coccolithophore species. These statistical link are much greater that the simple correlation between "δ – δ" values and $[CO_{2\,aq}]$, confirming a preponderant role of cell dynamics in the expression of the vital effect.~~

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 ~~(equilibrium)~~ 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.