Review to "Vanishing coccolith vital effects with alleviated CO_2 limitation" authored by Michäel Hermoso, I. Z. X. Chan, H. L. O. McClelland, A. M. C. Heureux, and R. E. M. Rickaby

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 $\delta^{18}O$ 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 12 $mmol \cdot L^{-1}$. Measured $\delta^{13}C$ and $\delta^{18}O$ 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 $\delta^{13}C$ and $\delta^{18}O$ values. Then, the authors try to interpret found correlations for $\delta^{13}C$ and $\delta^{18}O$ 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. 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

[1] "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."

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 CO_2 and calcite is precipitated from CO_3^{2-}. 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" (^{12}C and ^{13}C) 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 $\delta^{13}C_{DIC} \sim \delta^{13}C_{HCO_3^-}$) is presented and not $\delta^{13}C_{calcite}$ - $\delta^{13}C_{CO_2}$. $\delta^{13}C_{calcite}$ - $\delta^{13}C_{DIC}$ does not deviate strongly from 0. $\delta^{13}C_{calcite}$ - $\delta^{13}C_{CO_2}$, in contrast, would (not shown) strongly exceed zero. It hence follows that calcite was most likely built from external HCO_3^- and not CO_2.

Major concerns

- The heart piece of the work, i.e. the data set, is not presented. Furthermore, no error bars (how many replicates?) are presented in the figures. Tables that list carbonate systems and results should be given. 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.

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

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

- I am not sure, but the correlation between $\delta^{13}C$ and $\delta^{18}O$ and this DCUt index (calculated from $[CO_2]$) might originate from the correlation between $\delta^{13}C$ and $\delta^{18}O$ and $[CO_2]$ (latter correlation in most cases higher). This should be tested statistically.

- I am of the opinion that the title statement "vanishing coccolith vital effects with alleviated CO_2" is misleading and does not follow from the presented work.

- I am of the opinion that a profound discussion on cellular carbon fluxes when just having $\delta^{13}C$ and $\delta^{18}O$ data (without the corresponding data of the organic phase or particulate organic and inorganic carbon fixation rates) is not possible.

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 12 mmol·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 14 mmol·L^{-1} for *E. huxleyi* or *C. pelagicus*?

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

- 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.

- 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.

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 pCO_2" could otherwise be misunderstood.

- "As varying the availability of ambient DIC (primarily CO_2)": 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 HCO_3^- (you can see that in your data)!!! The carbon source of photosynthesis can be CO_2 and/or HCO_3^- (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). CO_2 limitation (in E. hux) occurs at CO_2 concentrations below 10 μmol·L^{-1} (cf. e.g. Bach et al. [9]).

- You should compare your carbonate system ([CO_2], [HCO_3^-], DIC, and total alkalinity (TA)) to ancient conditions here.

p. 15840

- l. 1f.: Why do you first remove all DIC from the water? You measured (and calculated) δ^{13}C ...

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

- You should really add a table with the carbonate systems (incl. TA, DIC, CO_2, HCO_3^-, CO_3^{2-}, and maybe [Ca^{2+}]) and also mention salinity. If you do not want to put it into the main document, you should definitely provide it as appendix.

- 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.

- 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...

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

p. 15841

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

p. 15842

- eqn. (2): How reliable are the calculations of $\delta^{18}O_{inorg}$ and $\delta^{13}C_{inorg}$? It would make more sense to me to calculate ε values that relate the calculated isotopic calcite signal to the isotope value of the external carbon source (i.e. relationship between $\delta^{13}C_C$ and $\delta^{13}C_{HCO_3^-}$ in terms of calcite, ε is close to 0 (Rost et al. (2002)) $\rightarrow$ almost no fractionation between ext. HCO_3^- and calcite $\rightarrow$ HCO_3^- seems to be the carbon source of calcite ...). Well, in fig. 2 you give the Δ between $\delta^{13}C_C$ and $\delta^{13}C_{DIC}$...

- I don't understand why you give the difference between $\delta^{13}C_C$ and $\delta^{13}C_{DIC}$ (which is close to $\delta^{13}C_{HCO_3^-}$) and not the difference between $\delta^{13}C_C$ and $\delta^{13}C_{CO_2}$, when you assume that CO_2 is the external carbon source. When plotting the latter value, you could see that ($\delta^{13}C_C$ - $\delta^{13}C_{CO_2}$) becomes much higher, i.e. the offset to zero increases strongly ($\delta^{13}C_{CO_2} \approx \delta^{13}C_{HCO_3^-}$ - 10)! This shows that HCO_3^- is the prime carbon source of calcite, not CO_2.

- 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 H_2O molecule.

p. 15842

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

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.

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 12 mmol DIC·L^{-1}, the value of E. hux is below the inorganic one.

p. 15846

- DCUt as index for "internal carbon pool": Who's interpretation is that?
- CO_2 cannot be the only carbon source of the cell! cf. e.g. your own data set. ...
- Your interpretation of DCUt is strange, in my opinion.
- My interpretation of DCUt is that it indicates how much CO_2 may be used for photosynthetic carbon fixation. The remaining carbon demand (of photosynthesis) would have to be covered by HCO_3^-. "$\mu \cdot$ volume"[2] gives an indicator for the photosynthetic (!!) carbon demand of the cell. "$[CO_2] \cdot$ surface-area", in contrast, is used as indicator for the potential to take up CO_2 via the surface-area (diffusive CO_2 uptake is[3] dependent on

[2] or better: "(POC per cell after cell division) · division rate per day"
[3] besides internal $[CO_2]$ and membrane permeability

external CO_2 concentration and the surface-area). Depending on which carbon species is used for photosynthesis, external CO_2 or HCO_3^-, 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.

- I think that $\mu \cdot$ volume $\cdot$ surface area^{-1} does not change much in comparison to $[CO_2]$. When altering light or nutrient conditions this may be different though. However, you should test the significance of the correlation between δ and DCUt cs. δ and $[CO_2]$.

- l. 10 CO_2 is not the carbon source of calcite

- E. hux is not the only alga that can use CO_2 as well as HCO_3^- for photosynthesis. This is a common feature, although it may be the only coccolithophore species for which a shift towards CO_2 usage at high $[CO_2]$ has been measured.

- l. 25: Why should cells take up more HCO_3^- at high $[CO_2]$? 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 CO_2. Kottmeier et al., in contrast, rarely changed HCO_3^-.

- "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 (+ CO_2 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 ...

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?
- DIC is the sum of CO_2, HCO_3^-, and CO_3^{2-} (+ H_2CO_3) and I suppose the cell reacts to all C species differently, depending on the type of import mechanism for instance.
- l. 12: reference to Fig.
- secs. 3.4.1 and 3.4.2: As mentioned above already, I think the correlations may be better with CO_2 than with DCUt.

p. 15848

- sec. 4.1: How do you explain that $\delta^{13}C$ and $\delta^{18}O$ both increase or both descrease for one species? Isn't that counter-intuitive when considering CO_2 in the external medium (equilibrium) being depleted in ^{13}C, but enriched in ^{18}O compared to HCO_3^-?

p. 15849

- l. 2/3: Inorganic or organic precipitation? Did Anning et al. do precipitation experiments? Watkins and Hermoso: O isotopes, not C isotopes?
- l. 5: "co-evolution of DIC": I don't understand that. A reference is missing
- l. 22: No. We did not separate photosynthesis and calcite precipitation for DIC sourcing in our early coccolithophore modelHoltz et al. [8]. As a result, we find that CO_2 and HCO_3^- interconversion inside the cytosol is low, leading to a separation between CO_2 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 $[CO_2]$ with the elevated uptake of HCO_3^- for photosynthesis – which strongly opposes the idea of separated DIC sources for photosynthesis and calcite precipitation!

- 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.

- 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...

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))

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

- l. 13: Langer et al. (2009) used carbonate systems with DIC $\sim 2\,mM$ 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.

- 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?

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

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

- I miss the statement that CO_2 is isotopically depleted in ^{13}C 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: CO_2 diffuses across membranes. Thus, at high DIC, where $[CO_2]$ is very high also, CO_2 enters the cells and brings in a lot of ^{12}C (comp. to ^{13}C). Most organic carbon might be built from CO_2 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?

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 ...

- l. 13: The data of Langer et al. (2006) are not comparable to your data set. DIC varies around $2\,mmol{\cdot}L^{-1}$, pH is lower at high $[CO_2]$ 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

- l. 22: Changes of $\delta^1 8O$ (changes in which which direction?) may originate from increased proportion of HCO_3^- over ....

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

- 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 ^{12}C and ^{13}C necesary) can alter the isotopic signal...

- 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.

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
l. 10 mMk_{sw}^{-1}?

p. 15841
. 15-19: Which standard did you use for δO? L. 15: V-PDB; l. 19: V-SMOW – I am confused. Cf. next page, l. 6-8.
l. 25: Here, you should first introduce the calculations of $\delta^{13}\mathrm{C}_C$ and $\delta^{8}\mathrm{O}_C$. – where are the values?
l. 26: "offset of coccolith calcite from inorganic" (add) calcite.

p. 15842

l. 19: "per day ($\mu \sim 0.7\,\mathrm{d}^{-1}$)": 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 $\mu > 1$.

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

p. 15843

l. 1: $\delta^{13}\mathrm{C} = \delta^{13}\mathrm{C}_C$?

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

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

l. 1-3: do you speak about the vital effect or $\delta^{13}\mathrm{C}_C$?

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

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

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

p. 15844

l. 1: it can be, not been

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

l. 17-18: is this a sentence?

p. 15844

l. 20: "become relatively larger"

p. 15845

l. 3: wrong Fig. cited?

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

p. 15848

l. 19: “such contrasting responses”: reference to which responses?

p. 15850

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

l. 1/2: The sentence is weird.

l. 2: “coccoliths” should read coccolithophores

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

References

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