Dear Pr. Middelburg,

We thank again Reviewer Hongrui Zhang for reviewing the new version of our manuscript and are pleased that he is satisfied with the answers and suggested changes.

We would like to sincerely thank Reviewer #3 for taking the time to read and comment on our manuscript. We are pleased to hear that they found the data interesting and appreciated the way it was analyzed. We also appreciate their constructive comments (highlighted in orange) and address them point by point below (in black). The corresponding changes to the manuscript, primarily additions, are shown in blue.

I could not find explanation how the pH was actually measured. Only the mentioning that it was on the total scale. Did you use an electrode with TRIS seawater buffers for calibration or spectrophotometry? Also, what is the uncertainty of this measurement and how would it propagate for calculated pCO2?

We did not explicitly mention how the pH was measured in the original manuscript, and we thank the referee for pointing out this omission. The pH values were determined using electrometric measurements with a Mettler Toledo pH InLab® Routine pro-ISM electrode. Calibration was performed using a TRIS buffer solution (T34, Dickson Lab, see: CO2crms.ucsd.edu). The uncertainty of pH measurements with this electrode is estimated at 0.03 pH units (Meinrath and Spitzer, 2000). When propagated to the pCO_2 estimates using the CO2SYS spreadsheet, this uncertainty results in an error margin of ±15.5 ppmv for the pCO_2 of culture condition 1 (8.29 pH units and pCO_2 of 200 ppmv) and ±96.9 ppmv for culture condition 2 (7.55 pH units and pCO_2 of 1400 ppmv). These uncertainties were already accounted for in the final $\Delta\delta^{13}C_{small\text{-}large}$ / CO_2 equations through Monte Carlo simulations.

Meinrath, G., Spitzer, P. (2000). Uncertainties in Determination of pH. Mikrochim Acta 135, 155–168. doi.org/10.1007/s006040070005.

Changes in the revised manuscript (Section Materials and Methods) :

The pH values were checked by electrometric measurement using a Mettler Toledo pH InLab® Routine pro-ISM electrode. Calibration was performed using a TRIS buffer solution T34 (Del Valls & Dickson, 1998).

Del Valls, T.A. and Dickson, A.G. (1998). The pH of buffers based on 2-amino-2-hydroxymethyl-1,3-propanediol (‘‘tris”) in synthetic sea water. Deep-Sea Res. 1 (45), 1541–1554.

The residual errors (also accounting for pH/CO_2 uncertainties) are evaluated through Monte Carlo analysis code with 1,000,000 iterations and an uncertainty of 0.17‰ for the differential vital effect between small and large coccolithes $\Delta\delta^{13}C_{small\text{-}large}$ (1σ).

Cell size is probably not the only factor influencing the degree of vital effects, as otherwise there should be similar ones found for H. carterii as more or less the same size as C. braarudii (see also next point).

We agree with the second comment from referee 2. The importance of collectively considering growth rate, PIC, POC, and the PIC:POC ratio in the expression of vital effects is already acknowledged in the manuscript (see lines 55 and 84). There is indeed no direct correlation between cell size and the magnitude of vital effects. Consequently, *H. carterii* and *C. braarudii* do not exhibit the same vital effect, as these species have distinct growth rates and PIC/POC ratios.

Line 55 reads : *However, when calcite is biomineralised intracellularly, biological parameters such as growth rate, cell size, and the PIC/POC ratio - which refers to the distribution of carbon between particulate organic carbon (POC) and particulate inorganic carbon (PIC) produced by calcifying organisms - also influence this fractionation (Dudley et al., 1986; McClelland et al., 2017; Rickaby et al., 2010).*

Line 84 reads : *Modelling studies fed by culture data have identified and quantified the main forcing parameters behind the magnitude of carbon isotope vital effect in coccolith calcite: growth rate, cell size, the partitioning of CO_2 in particulate inorganic matter and particulate organic matter (PIC/POC ratio), among other ancillary parameters (McClelland et al., 2017)*.

For both quotations (and elsewhere in the ms), we thus believe we have already acknowledged that not only cell size was at play for the expression of the isotopic vital effects, but also growth rates and PIC/POC ratios.

Metabolic responses (growth and carbon fixation rates) of coccolithophores to increasing CO2 and decreasing pH follow optimum curves. If such curve is actually observed or not depends on the CO2 range chosen. The sensitivity to low CO2 levels (left hand side of the curve) and high proton concentrations (right hand side of the curve) is species and even strain specific (e.g. Langer et al. 2009). Furthermore, the response is modulated by other environmental factors such as light and temperature (e.g. Sett et al. 2014, Gafar et al. 2018). Hence, the pCO2 range within which vital effects are being displayed or not will vary. It is therefore unlikely, that a single transfer function derived for a particular combination of abiotic culturing conditions will be sufficient to reconstruct paleo pCO2 in a variable paleo environment (temperature, light, ...). Furthermore, and on top of potential strain-related vital effects, it would need to be shown that the off-set between small and large species is constant for different temperature and light conditions. All these caveats should properly be discussed and conclusions should be more cautious.

We agree with this view point. In this study, only the carbonate chemistry of the culture medium was changed, keeping other parameters constant: light irradiance and temperature as pointed by the Reviewer. Previous studies have also revealed a strain-specific biogeochemical response, especially for the morphotypes of *Emiliania huxleyi*. We will make these caveats more explicit in the revised manuscript.

In Section *4.3.2 Palaeoclimate application of carbon isotope culture data*, we already stated the following sentence: *One promising research avenue would be the study of the co-variation of CO_2/pH levels crossed with temperature changes in new culture campaigns. This approach could have the potential to revel the synergistic effect of discrete various controls on cell growth rates and refine the biogeochemical understanding of the vital effects*.

We will include a preliminary sentence at the beginning of the discussion (which will be recalled in the conclusion) so that the reader can appreciate the complexity of reproducing the natural environment (both geographically temporally) in experimental studies. We will include the suggested references.

Thus, we will add: "In this study, we chose to perturb only the carbonate chemistry of the culture medium in which the cells grew. It is important to remember that other environmental factors, such as light irradiance and temperature, also influence cellular growth and the magnitude of vital effects (Langer et a., 2009; Sett et al., 2014; Hermoso et al., 2016; Gafar et al., 2018)".

&

"The extent to which our biogeochemical calibration can be applied to wild and fossil coccoliths depends on the (paleo)environmental context. In these experiments, only the biogeochemical responses of monoclonal strains have been examined, while variations in light irradiance and temperature may interact with changes in the carbonate chemistry of water masses. In the future, a broader range of strains and additional physico-chemical parameters must be studied, integrating these factors to develop a unified response through modelling".

The array of pH conditions applied in the cultures is appreciable (7.55 – 8.29 pH units) and encompasses, to our best knowledge, most of the variation of this parameters in present day oceans. The investigated pH range also covers the documented variations of oceanic pH over the last 66 MYrs: 7.5 in the Eocene, 8.2 in the Pleistocene (Rae et al., 2021). The full spectrum of the pH/CO_2 forcing on physiological and isotopic responses by coccolithophore cells is therefore documented here.

Rae J.W.B., Zhang Y.G., Liu X., Foster G.L., Stoll H.M. and Whiteford R.D.M. (2021). Atmospheric CO2 over the Past 66 Million Years from Marine Archives. Annu Rev Earth Planet Sci, 49, 609-641. https://doi.org/10.1146/annurev-earth-082420-063026

Comparing the two transfer functions to derive paleo pCO2 from inorganic d13C measurements of large and small coccoliths shown in Figure 9 highlights the complications outlined above. For the same pCO2, they show differences of 1-2 per mille, meaning that, based on isotopic measurements, the reconstructed pCO2 would be off by a few hundred ppmv. Such high uncertainty should be acknowledged.

As mentioned to the reviewer 1 during the first round of peer-review, it is noteworthy that the two calibrations match pretty well, at least to first order (see Figure A1 for data over the entire pCO_2/pH interval). The offset between the two studies can indeed originate from the above-mentioned points and/or the experimental setup. Replicated measurements within a much more restricted spread of values in our study gives confidence to the calibration (once again, in the conditions in which the cultures were performed). Our study has the advantage of having more measurements at low pCO_2 compared to the work of Rickaby et al., 2010. This was the rationale for our culture work here. However, we acknowledge that there is a difference up to 1.5 ‰ between our calibration and that of Rickaby et al., 2010 at low pCO_2 and high pH. The differences between the two calibrations highlight the idea exposed by referee 2 (in their third remark), i.e. we need more data from multi-stressor experiments / strains to refine the transfer equation. Implementing very dilute semi-continuous culture batch is essential for generating such biogeochemical dataset and avoiding a reservoir effect.

We propose adding the following paragraph at the end of Section 4.3.1:

A comparison of our calibration with the data published by Rickaby et al. (2010) in Figure 9 reveals a discrepancy of up to 1.5‰ under the lowest pCO_2 / highest pH conditions. Explaining this difference is challenging, particularly because the studies were conducted under different conditions, involved different strains, and included only a few data points in this low CO_2 range, as documented by Rickaby et al. (2010). This observation may call into question the feasibility of achieving a species- and environment-integrated response in coccolithophores (see further discussion in Section 4.3.2).