

Author responses to referee comments on “Physiology regulates the relationship between coccosphere geometry and growth-phase in coccolithophores” by Rosie M. Sheward et al.

We kindly thank both reviewers and the Editor for their positive, insightful and constructive comments on our manuscript and we are pleased that the manuscript was considered to be well-written, clearly structured and accessible to non-specialists, with well-reasoned rationale and a high-quality dataset.

We have carefully considered all comments and suggestions and hope that we have satisfactorily addressed each point raised. The main points that we have addressed are as follows (see details in specific responses):

Applicability and significance of work: Both Reviewers questioned how common the occurrence of coccospheres is in the fossil record and therefore how broadly applicable our results may be. Our wider research demonstrates that coccospheres are uncommon but not so rare (documented from a range of ages, ocean basins and latitudes) that our approach would only be applicable in very few cases. We have now emphasised our comments on applicability in the introduction, discussion and conclusions.

Correction of Table 1: We thank Reviewer #1 for their diligence in identifying inconsistencies between the data in Table 1 and in the text. We have checked all reported values for accuracy and have changed values in Table 1 and in the text where necessary.

Additional Supplementary Table: In light of suggestions from Reviewer #1, we have prepared a Supplementary Table (Table S1) to report growth rates and minimum, mean and maximum coccosphere geometry for Exponential-phase and Non-exponential-phase days separately to complement the ‘full dataset’ coccosphere geometry summarised in Table 1.

Growth phase, growth rate and fitness comments: We have carefully considered the comments of Reviewer #1 concerning interpreting growth information in the fossil record based on growth *phase* results rather than growth *rate* results. In light of specific comments, we have adjusted our phrasing for clarity and to avoid misleading word choices in several places (Ln 67-68; Ln 368; Ln 375-379). We have also added additional comments in the discussion on the value of other experimental approaches in addressing growth rate and coccosphere geometry in future investigations to further advance our work.

Statistical methods: We have added a ‘Statistical Methods’ sub-section to the Methods following the suggestion of Reviewer #2, including bootstrap analysis of our linear regression analysis, which we agree improves the high quality of our extensive dataset.

Below are the detailed author responses to the individual comments made by Reviewer #1 and #2.

Response to Reviewer #1

General comments:

1: The manuscript is well written and overall clearly structured, although the results and discussion sections are not clearly separated with some results discussed in the results section and some new data (μ , PIC) introduced in the discussion.

Author response: We introduce ‘new’ analysis into our discussion about the wider implication of our results for calcification rates, building on previous discussion about coccosphere geometry and growth phase. The exponential growth rate data is presented in the Results: Sect. 3.1 – Growth rates.

We now additionally include the range of growth rates during exponential and non-exponential growth days in this section and summarise this in a new Supplementary Table. The PIC per cell and PIC production data is presented for the first time in Sect. 4.4 as it is not addressing the primary aim of the study. However, it will now be reported within the results section.

2: There is a lack of consistency between described results and presented data (especially table 1)

Author response: We apologise for the errors in Table 1 and have now checked for and corrected any misreported values in the text and Table 1, see specific comments and revised data in Table 1.

3: Not all data described in the methods/presented in table 1 are discussed e.g. POC data.

Author response: We have removed POC data from Table 1.

4: I wonder what the range of applicability is. Do the authors have any knowledge on how common it is to find intact coccospheres rather than single coccoliths in the fossil record? Is there any data available for the species presented in the current manuscript?

Author response: The presence of coccospheres in sediments is considered atypical, though the authors of this study are involved in a project specifically investigating Paleogene coccospheres (manuscript in preparation) that targets sediments with higher numbers of coccospheres than usual, ~20 to >300 per standard smear slide (see Gibbs et al., 2013 and Bown et al., 2014). So far, we have documented and measured 4,388 individual coccospheres spanning more than 40 Paleogene species and 9 families, including important extant families. Therefore, coccosphere geometry is potentially relatively widely applicable in the geological record.

We explain our reasoning for choosing *Calcidiscus* and *Helicosphaera* species to supplement *Coccolithus* data in the Introduction (L 76-95). *Coccolithus* is an exceptionally long-lived genus, first originating ~66 Ma, and the species *Coccolithus pelagicus* has arguably existed for roughly the same length of time. It represents a large proportion of our fossil coccosphere dataset (~1800 coccospheres to date; Sheward et al., in prep). In the Paleogene, where we are currently focusing our fossil coccosphere studies, *Calcidiscus* and *Helicosphaera* are less abundant contributors to the overall assemblage and we do not have detailed fossil coccosphere data for them, although we do have some Miocene fossil coccospheres of *Helicosphaera* and *Calcidiscus* spp. coccospheres from the Middle Eocene. However, these genera become increasingly abundant into the Neogene and towards the present. Their robust coccolith morphologies and coccosphere architectures give them a comparably high preservation potential, similarly to *Coccolithus*. The majority of the fossil coccospheres we see are extinct species, but information about taxa in the same genus or family is still highly valuable.

In terms of applicability outside these genera, we detail in Sect. 4.3, L 346-354 how our methodology using C_N thresholds to investigate qualitative variability in growth phase could be applied to any extinct taxa provided there was an abundant fossil coccosphere geometry dataset to use.

5: The hypothesis that coccosphere geometry can give information on population fitness is quite bold. In fact, the authors are very well aware of this fact and discuss the difference between growth phase and growth rate. The authors write themselves (L 301-303) "growth rates would not necessarily be expected to influence coccosphere geometry in the same way as a shift in growth phase". I would agree, but this limits the applicability of coccosphere geometry to the fossil record. To relate coccosphere geometry and nutrient availability in a geological setting, it is necessary to (at

least) look at whether nutrient-induced changes in steady state i.e. continuous growth rates have an effect on average coccosphere geometry. The authors tried to address this by growing the species at different temperatures, which failed to induce a significant range in growth rate. The concept of “growth phase in the fossil record” is difficult because growth phases are of very short duration in nature. Over long time periods, steady state or maximum exponential growth rates become important. It is therefore misleading to relate growth phase to the fitness of a population (e.g. L 68; L 378) because all populations undergo different growth phases. The discussion would benefit from including some thoughts on testing this hypothesis further e.g. by using chemostats.

Author response: We did not intend for our phrasing to lead readers to consider our discussion as presenting a ‘proxy for fitness’. Biological fitness describes population reproductive success that is usually measured as growth rate in experimental phytoplankton studies (e.g., Collins et al., 2013) and cell size and calcification are also considered to be important fitness-related traits (e.g., Lohbeck et al., 2012; Benner et al., 2013; Jin et al., 2013). As our results produced no obvious correlation between growth rate and coccosphere geometry, we currently cannot use coccosphere geometry to estimate a specific growth rate in field populations or fossil assemblages. However, as we say in L 59-61, growth phase describes two different states, one of which is of slowed to zero growth caused by nutrient paucity. Therefore, analysing the coccosphere geometry of populations/assemblages and its variability through time using the method we present identifies intervals when population coccosphere geometry is more representative of such a slowed growth state or conversely rapid, exponential growth. This type of growth information is not measurable from typical coccolithophore remains in the fossil record (usually just loose coccoliths), so it is the first indicator of growth of any form from intact coccospheres.

In light of the reviewers comments, we have re-phrased L67-68 and L375-379 so that they do not imply growth phase as a fitness trait, rather that interpretations of growth phase in field or fossil population is an important first step in retrieving any sort of indication of fitness traits in the fossil coccolithophore record.

We now add a section in the discussion following on from the end of L368 to address the valuable additional perspective that using continuous or semi-continuous approaches would provide, and that this approach should be used to specifically investigate the effect of a range of steady-state growth rates on coccosphere geometry, which would greatly strengthen future investigations of growth in the fossil record.

6: It could be made clearer that it is only the average coccosphere size and CN that shifts, whereas the range is the same in both growth phases (Figure 3). Although Figure 4 looks very impressive, it creates the idea that in exponential phase “all cells” are small, whereas in stationary phase “all cells” are large. However, figure 3 makes it clear that both stages span the same size range and there is only a slight (though significant) shift in the average size.

Author response: Fig. 4 is largely to demonstrate visually that coccosphere geometry is very variable within a species, as typically only mean values and standard deviations are referred to in published studies and we have rephrased the caption of Fig. 4 to make this very clear. By referring to the ‘recently-divided’ and ‘ready to divide’ types of cells, we are highlighting to the reader the ‘end-member’ type of coccosphere geometries used in estimating the proportion of the population that has recently undergone cell division, which will be higher during exponential phase when the time between cell divisions is shorter. We show this in Fig. 5 for exponential days and non-exponential days of growth in these culture experiments and selected field samples experiencing characteristics of ‘bloom’ and ‘non-bloom’ conditions.

To improve clarity, L252-254 have been re-worded to more clearly state that there is an increased proportion of cells in larger \emptyset or C_N size classes respectively, and a shift in mean \emptyset and C_N (which has been tested statistically). Section 3.3 of the results describing Fig. 3 is now more explicit, making it much clearer when changes in mean \emptyset or C_N are referred to, relative to overall changes in the histogram shapes. We have also added that minimum \emptyset and C_N shifts towards larger values in the non-exponential-phase data (\emptyset increase of 0.77 to 2.29 μm , representing a species-specific 6.2 to 24.5 % increase on exponential-phase minimum \emptyset , and a C_N increase of 1 to 4 on minimum exponential C_N , representing a species-specific increase of 10 to 50% on minimum exponential C_N). This data is now shown in the Supplementary Table.

Specific comments:

7: L 14-16 - “however, to realize the potential of this archive requires an understanding” needs to be rephrased.

Author response: Re-phrased as “However, to realize the full potential of this archive for (paleo-)biology and biogeochemistry requires an understanding...”

8: L 36 - why do the authors use a threshold size of 63 μm , instead of e.g. 200 μm , the threshold for microplankton? Does this relate to the max. size of coccolithophores?

Author response: Nannoplankton and nannofossils are defined by Lohmann (1909) and Young et al. (1997) as ranging between 2 and 63 μm and the 63 μm threshold is employed by micropaleontologists wishing to physically separate the calcareous nannoplankton fraction (coccolithophores and other nannoplankton) from other microfossil groups within a sediment sample. The nannoplankton range is considered by some authors to be between 2 and 20 μm (e.g., Sieburth et al., 1978; Finkel et al., 2010). However, coccolithophore cell/coccosphere sizes regularly exceed this, with the approximate maximum coccosphere size in modern coccolithophores $\sim 53 \mu\text{m}$ in *Coronosphaera* sp. (O'Brien et al., 2013). Using a 200 μm threshold would be suitable for phytoplankton in general, but may be misleading about the maximum size of coccolithophores.

9: L 37-38 - what do the authors mean by “the biomass that sustains the wider diversity of marine life at higher trophic level”? I would argue that the (genetic) diversity of photosynthesizing plankton surpasses that of higher trophic levels.

Author response: We have revised the phrasing for clarity. We simply meant that the biomass produced by photosynthesizing plankton fuels all the marine life at higher trophic levels.

10: L 93 - should it be “classified into separate families” rather than “in”?

Author response: We have changed the sentence to now read “classified into separate families”

11: L 105 - please check the calculation of daily photon flux; it should be half that value; maybe the calculation was carried out using 24-h light?

Author response: The reviewer is correct that daily photon flux should be half of the value stated, it was calculated for a 24 hour ‘day’ without accounting for the ‘daylight’ period being only 12 h of that. Values of 75 – 90 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, under a 12 h photoperiod, 75 = 3.24 and 90 = 3.89 $\text{mol photons m}^{-2} \text{d}^{-1}$.

12: L 106 - the authors could consider mentioning up front that the different temperatures failed to give a significant range in cell division rates and therefore all data was pooled.

13: L 108-110 - I would add a table as supplementary information with μ (rather than just mentioning the range in the discussion) and coccosphere geometry parameters for each temperature experiment.

Author response: All of the μ and coccosphere geometry parameters for every experiment day are provided as a pangeae.de datafile. For completeness, a new Supplementary Table now presents mean, min and max C_L , C_N , \emptyset , Θ for exponential and non-exponential days and mean exponential μ for each temperature experiment.

Cont. from above: The authors state that there were no (significant?) differences among the temperature treatments. However, they have used only two replicates for each temperature. How was this tested?

Author response: We state L 299-300 that “exponential growth rates were not that sensitive to the temperature range we applied (*C. quadriperforatus* $\mu_{\text{exp}}=0.30-0.44 \text{ d}^{-1}$; *C. leptoporus* $\mu_{\text{exp}}=0.31-0.44 \text{ d}^{-1}$; *H. carteri* $\mu_{\text{exp}}=0.28-0.45 \text{ d}^{-1}$.” The reported values are the minimum and maximum mean exponential growth rates across the four temperature conditions for each species. We do not statistically compare the two replicates at each temperature. When coccosphere geometry data from exponential experiment days was analysed against the mean exponential growth rate of the experiments there was no statistical correlation that would suggest an obvious relationship between growth rate and any coccosphere geometry parameter that was measured.

14: L 114-115 - please give nitrate and phosphate concentrations of the medium as this is important for interpreting final cell concentrations. The authors give references to the K/20-medium, but following Daniels et al. (2014), nitrate concentrations would be 28.8 μM and phosphate 1.8 μM , following Gerecht et al. (2014), concentrations would only be 16 μM nitrate and 1 μM phosphate. Šupraha et al. (2015) presented data on the same strain of *Helicosphaera* used in the present experiment which entered stationary phase at ca. half the cell concentration (15500 cells mL⁻¹) as in the current study (growing on 1 μM initial phosphate; I therefore assume phosphate concentration was 1.8 μM ?)

Author response: We have modified L113-115 to now give details of the medium nutrients, as follows, and removed the reference to Gerecht et al. (2014) to avoid confusion as they used different concentrations:

“...added to 350 ml of sterilised and filtered natural seawater enriched with 28.8 μM nitrate and 1.8 μM phosphate (lower-nutrient K/20 medium, modified from Keller et al., 1987, following Langer et al., 2006 and Daniels et al., 2014).

15: L 115-119 - Daniels et al. (2014) grew dilute batch cultures and harvested in exponential phase, whereas the authors have used the same medium (?), but harvested in stationary phase. In fact, the cultures reached much higher cell concentrations (for *C. braarudii* 25.000 cells mL⁻¹ vs. max. 8.700 in Daniels et al. (2014)).

Author response: Yes, we used the same growth medium and the difference in maximum cell concentrations arises because Daniels et al. (2014) sampled in mid-exponential phase growth (to ensure truly exponential phase growth) not at maximum cell density (where cells are no longer in

exponential phase growth under a batch culture approach). Samples for cell counts and LM coccosphere geometry measurements in *Coccolithus* (Gibbs et al., 2013), *Calcidiscus* and *Helicosphaera* were harvested daily throughout the experiment, not just in the stationary phase.

16: The authors do not give information on the carbonate chemistry, but based on the high final cell concentrations, I would assume that there was a significant consumption of DIC (for comparison, in Gerecht et al. (2014), *C. braarudii* reached max. cell concentrations of 17550 cells mL⁻¹, which reduced DIC down to 1200 μM. A rough calculation and assuming a proportional response, at 25000 cells mL⁻¹, DIC would be reduced to ca. 900 μM which is assumedly limiting for growth (Bach et al., 2013). How much can this really be compensated for by passive diffusion into a bottle and then into the medium, which are both presumably slow processes? Similarly, Šupraha et al. (2015) presented data on a Mediterranean strain of *Helicosphaera* that entered stationary phase at 41.000 cells mL⁻¹ vs. the 30.000 in this study. At 41.000 cells mL⁻¹, carbonate chemistry was severely altered (and PIC quota of the single coccoliths was affected). A significant change in carbonate chemistry and ensuing DIC limitation does not directly affect the conclusions of the manuscript, also because PIC quota was not directly measured, but inferred from coccolith length measurements. However, L 115-119 is misleading in suggesting that carbonate chemistry was not affected as no data is presented to confirm this. On the contrary, the available data indicate that there was a significant impact (as outlined above).

Author response: We have now rephrased L 115-119 to avoid implying that the carbonate chemistry was unaltered, although we cannot report on specific changes as the carbonate system was not monitored. We thank the reviewer for pointing out that DIC could have changed over the course of the experiments and influenced growth and/or calcification and we agree that any change in the carbonate chemistry would not affect the conclusions that we draw from our results. The culturing flasks that we used have membrane caps that allow faster diffusive gas exchange than would occur in fully-sealed flasks as well as aerated and mixed each flask daily. Our light microscopy work did not show any evidence of coccolith changes e.g., malformation, that might suggest a detrimental affect of carbonate chemistry parameters on coccolith calcification during our experiments (see LM images in Figure 4).

17: L 127 - assumedly, formaldehyde was also added to *Helicosphaera* cultures for size measurements? Although the coccosphere is probably relatively stable, formaldehyde may lead to shrinking of the cell membrane, which could influence morphology measurements. Did the authors consider/check this?

Author response: Formaldehyde was only added to samples for cell counts and not to the samples for morphological analysis, which is why the formaldehyde is not mentioned in the Methods section 2.3: Coccosphere geometry.

18: L 135 - no need to mention that half of the filters were stored for SEM, if the data is not presented. Did the authors do any control measurements under SEM?

Author response: No SEM measurements were done for control and the comment is removed from the text.

19: L 143 - If coccosphere size is used as a (realistic) proxy for cell size, the terminology should be used more clearly. In the methods, coccosphere and cell size are presented as two different parameters, whereas in the abstract “cell size” is used more loosely.

Author response: The measurement of the interior of the coccosphere is termed as ‘cell diameter’ and the measurement of the exterior of the coccosphere is termed ‘coccosphere diameter’ (this is the measurement that would be taken from an SEM image), as shown on Figure 1 and described in the Figure caption, with the assumption that the cell fully fills the coccosphere. The method text L143 now says “...and cell size (\emptyset ; size excluding calcite covering that is assume to be equivalent to cell diameter)...” and the use of cell size in the abstract has been replaced with coccosphere size.

20: L 170 - as daily growth rates ranged between max. and zero, it is confusing to write “resulted in a modest range of daily and mean exponential growth rates”; remove daily

Author response: By this sentence we mean that both daily growth rates during the exponential days of the experiment (growth rates calculated between consecutive days) and the mean exponential growth rate calculated across all exponential days of growth show a modest range. The sentence has been changed to improve clarity.

21: L 170 - which days were used to calculate mean exponential growth rates?

Author response: Days during which cultures were growing at an exponential rate are as follows: *C. quadriperforatus* = up to and including Day 11 (22 °C and 20 °C), Day 16 (18 °C), Day 17 (16 °C); *C. leptoporus* = up to and including Day 15 (20 °C), Day 11 (20 °C), Day 17 (18 °C), Day 18 (16 °C); *H. carteri* = up to and including Day 11 (22 °C), Day 7 (20 °C), Day 15 (18 °C), Day 13 (16 °C), *C. braarudii* = up to and including Day 11. Mean exponential growth rates (μ) for each temperature experiment were calculated from cell densities where $\mu = (\ln N_1 - \ln N_0)/d$, and N_1 and N_0 are cell concentrations at the beginning and end of the exponential phase and d is the duration of the exponential phase in days. A statement to this effect has been added to the end of Section 2.2, L130.

22: L 182 - none of the values reported in the text for coccosphere diameter correspond to those presented in table 1 i.e. max value for *H. carteri* is 21, not 15 μm .

Author response: We apologise for the minor errors in Table 1. All of the data in Table 1 has now been checked for accuracy and the values in Table 1 and the text corrected where appropriate. L182 now reads:

“A considerable range in \emptyset is seen in all species; 13.8 μm to 24.4 μm in *C. quadriperforatus*, 9.4 μm to 20.9 μm in *H. carteri*, and 10.0 μm to 19.7 μm in *C. leptoporus*. This is a comparable \emptyset range to *C. pelagicus* (11.7 to 20.8 μm) but slightly less than the \emptyset range observed in *C. braarudii* (13.7 to 29.7 μm).”

23: L 187 - mean CN for *C. braarudii* is 14, not 11- 12 μm .

Author response: L187 now reads “In contrast, *Coccolithus* cells more typically have 11-14 coccoliths per cell, up to a maximum of ~20 coccoliths.” *Coccolithus* includes both *C. pelagicus* and *C. braarudii*.

24: L 188-189 - move information on large coccosphere in *C. leptoporus* up one sentence.

Author response: Sentence has been moved.

25: L 211 - according to table 1, CL varies by 8.0 μm in *C. quadriperforatus*.

Author response: The minimum C_L reported for *C. quadriperforatus* in Table 1 was incorrect and has now been changed in the Table (min $C_L = 5.67$). A range in C_L of 6.0 μm as stated in L211 is therefore correct.

26: L217-218 - I agree that there is no relationship between C_N and cell diameter. However, I do not understand this sentence. The range in both coccosphere diameter and CL is very broad. Also, the cultures are not synchronized in regard to cell division as large cells (“about to divide”) are also present in exponential phase and small cells (“just divided”) also in stationary phase; only the peak of the mean shifts (see figure 3).

Author response: We believe that the reviewer here meant to say “I agree that there is no relationship between C_L and cell diameter” rather than C_N , which does of course show a relationship. The statement ‘relatively restricted ranges of ϕ and C_L ’ refers to the larger range in C_L and ϕ that results when you combine data from multiple populations, which does produce a C_L ϕ relationship, as shown in Gibbs et al. (2013). We have removed the remark “...cell division is fully synchronized across cells...”. This referred to sampling at the same time every day to minimize variability in coccosphere geometry resulting from the cell division cycle, such that the population should be made up of a broadly similar proportion of cells in different stages of the cell division cycle between each (exponential-phase) day. L216 now reads “In our clonal populations, cells have a relatively restricted range in ϕ and C_L that have no statistically-significant relationship (Fig. 2i-l).”

27: L 235-239 - the way this is phrased is misleading; it sounds as if “all cells” in stationary phase contain numerous coccoliths whereas you still have small, recently-divided cells.

Author response: L235-239 have been re-phrased to say explicitly that we report changes in mean ϕ and C_N and that overall the distribution of cells within the population shifts towards larger size classes, as shown by the histograms in Figure 3 (no change in maximum ϕ or C_N).

28: L 254 - in the results the shift in coccosphere diameter is 0.55-0.7 μm (1.75 μm for *C. braarudii* (L 229); this does not amount to ca. 2 μm . According to my calculations, using mean diameter presented in L 227-228 and the above mentioned increases, there is a 3% increase in *Helicosphaera* coccosphere diameter, 4-5% in *Calcidiscus* and 9% in *Coccolithus*. Here, however, the authors write 10-12%. If the authors calculated differently/used different values, this needs to be made clearer in the text.

Author response: Here, L254 should have read “...up to $\sim 2 \mu\text{m}$ ” but we agree that this would present an over-approximation so L252-254 now reads:

“Across all four species investigated, the transition from exponential into non-exponential phase growth was clearly associated with a shift towards an increased abundance of cells with greater C_N (mean C_N increased by 1-3) and larger coccosphere sizes (mean ϕ increased by 0.6 μm in *H. carteri* and *C. quadriperforatus*, 0.9 μm in *C. leptoporus* and 1.3 μm in *C. braarudii*). This represents a significant increase of 4 to 7% on exponential-phase mean ϕ and a significant increase of 10 to 27 % on exponential phase mean C_N (t -test, $p < 0.0001$).”

29: L 278 - POC production could have been affected by DIC limitation.

Author response: We say that POC per cell (not POC production) could increase under nutrient limited conditions but we assume that this is what the reviewer is referring to. In this case, referring to the data of Šupraha et al. (2015) for two *H. carteri* strains, the POC cell⁻¹ of their Atlantic strain increased 43.6% under P-limitation (no significant change in DIC, 1220 to 1159 μmol kg⁻¹) and the POC of their Mediterranean strain that did experience substantial DIC change (1292 to 639 μmol kg⁻¹) showed a POC cell⁻¹ increase of 46.7 %, which is not vastly different (their Supplementary Table 2 and Table 3). Moolna and Rickaby (2012) also report *Gephyrocapsa oceanica* coccospheres to have an “unchanged” coccosphere diameter under a past high-CO₂ condition with approximately five times higher DIC levels than modern (11.3 mM DIC, Ø = 6.50 μm compared to 2.3 mM DIC, Ø = 6.56 μm). However, Rickaby et al. (2010) show that *C. braarudii* POC increases with increasing DIC. This would suggest that the effect of DIC concentration on cellular POC maybe species-specific. We inferred POC changes from measured cell size, whereas the POC to volume relationship may or may not remain constant depending on the macromolecular composition (i.e., a change in Ø may or may not equal a change in POC). However, the possible influence of DIC on cellular POC will be added to L278.

31: L 300 - in results, max. μ for H. carteri is listed as 0.45 d⁻¹.

Author response: μ = 0.45 d⁻¹ as reported in the results is correct and L300 has been changed.

32: L 300-303 - This argument is not convincing. If coccosphere geometry is to be a tool for looking at long-term (as this is the fossil record) changes in growth, then it is necessary to look at reduced physiological rates due to e.g. lower nutrient availability. It is not possible to look at long-term shifts in growth phase in the fossil record. If there is an increased percentage in large cells in a certain sediment layer, what does this mean? That this was a time of “nutrient limitation” so that more cells grew larger and stopped dividing? Although this hypothesis is intriguing, it is necessary to look for a link between reduced physiological rates and larger cells if this is to be applied as a proxy to the fossil record.

Author response: Here we are expanding the concepts demonstrated in Gibbs et al. (2013) into three further species to look at the effect of reduced physiological rates due to lower nutrient availability on coccosphere geometry in the context of nutrient depletion towards the end of a batch culture experiment, a widely employed culturing approach. As stated in response to a previous comment, future work could indeed supplement the presented dataset and analysis with data from a continuous or a semi-continuous approach and would provide valuable additional information to strengthen future investigations of growth in the fossil record of coccolithophores. This has now been added to the discussion. But we do not agree that this is the only way to examine coccosphere geometry changes with growth phase to look at long-term shifts in growth phase in the fossil record, or that this detracts from the research, analysis and interpretation presented.

In answer to the reviewer’s comment “If there is an increased percentage in large cells in a certain sediment layer, what does this mean?” we show in Section 4.3 L 344-345 and Figure 5 that an increased percentage of coccospheres with a C_N typical of ‘ready to divide’ cells (i.e., greater than the 90th percentile of the C_N data) would be indicative of less favourable conditions for growth, where the population has shifted towards the coccosphere geometry characteristics of a population growing non-exponentially. To attribute such a response solely to nutrient availability would be an unrealistic simplification, rather we consider it a response to a ‘poor growth environment’ that could include any

factor or combination of factors that act to slow population growth. The modern field data presented by Gibbs et al. (2013) and shown in Fig. 5 contrasting the mean coccosphere geometries of bloom and non-bloom communities lends real strength to this approach and interpretation. This approach was also demonstrated across the PETM in O’Dea et al. (2014; their figure 1f) where the percentage of coccospheres exhibiting ‘slowed division’ (based on C_N) remained relatively constant in *Toweius* throughout the interval “indicating that high levels of cell division are maintained across the PETM” whereas coccosphere geometry indicated slowed cell division in *Coccolithus* across the same interval.

Our data supports that coccosphere geometry (C_N and \emptyset) can be used as a **qualitative** indicator of changes in growth state but we do not claim to be able to quantitatively determine growth rates or other physiological rates directly from fossil coccospheres. Our approach is presently the only method that can be used to access any degree of growth information in the fossil record. We make the relative limitations of this approach to field or fossil samples very clear - that “growth phase can be estimated” (L 355) and that “we must be clear that the environmental and growth signal recorded in field populations is always more complex than any laboratory experiment results” (L 363-364) before listing considerations that would affect the signal observed in L364-375.

33: L 310 - in this study, coccolith length is independent of growth phase so this factor of coccosphere geometry should be removed here.

Author response: To improve clarity, L310 has been re-phrased, as \emptyset , C_L and C_N are species specific, but \emptyset and C_N respond identically to growth phase changes. It now reads:

“A notable finding of this study is that coccosphere geometry (coccosphere size, coccolith length and coccoliths per cell) is species-specific but \emptyset and C_N responds identically to growth phase across four different species of *Calcidiscus*, *Coccolithus* and *Helicosphaera*.”

34: L 416 - Gerecht et al. (2015) show that growth rate determines calcite production in *C. braarudii*: PIC production is reduced by ca. 50% due to the 50% reduction in growth rate.

Author response: This reference has been added to L416.

35: L 420 - (*Toweius*) *pertusus* needs to be cursive

Author response: Corrected.

36: Table 1 - is this all combined data i.e. exponential and stationary phase? Table 1: check values Max PIC for *C. braarudii* (lower than Mean) Table 1: there is no mention in the methods as to how POC per cell was calculated, nor is it presented in the results or discussed in the rest of the paper.

Author response: All of the data for Table 1 has been checked against the original data and corrected where appropriate. An additional supplementary table now summarises coccosphere geometry separately for exponential and non-exponential days as well. POC data has been removed from Table 1 as it is not discussed in the manuscript.

37: Figure 2 - check frequency values for right y-axis.

Author response: Corrected from 50 to 5.

38: Figure 3 - How is “early stationary-phase growth” defined i.e. which daily growth rate is still considered exponential?

Author response: Early stationary phase growth describes informally the day(s) immediately following the departure of a plot of cell ml^{-1} vs. time data from an exponential curve fitted to definitely exponential days of growth. To avoid confusion and maintain consistency, the use of early stationary-phase growth in the description of Figure 3 in the text has been changed to “non-exponential”.

39: Figure 4 provides convincing evidence that coccosphere geometry data can be obtained by POL. I wonder, however, why SEM was not used? This would supply more accurate measurements of coccosphere diameter and especially of CL and equally adequate estimates of CN. It would not provide cell diameter, but the authors do not discuss cell diameter in the text. Cell diameter should be mentioned in the results as it is included in table 1, even if only as a short sentence e.g. cell diameter followed the same trend as coccosphere diameter (?).

Author response: We use LM for this study for a number of reasons. 1) It is very fast to prepare and analyse a large number of samples. The observation of coccospheres (culture, plankton or fossil) can be very time consuming using the SEM. 2) These species have morphologically ‘robust’ coccoliths and coccospheres, that is to say that the edges of the coccosphere (internal and external) and coccoliths (under polarised and plane light on the cross-polarised LM) are very clear. 3) C_N can be broadly estimated from SEM but LM allows us to count each individual coccolith in the coccosphere and this therefore highly accurate in comparison. 3) The LM reveals both external and internal dimensions of the coccosphere, whereas the SEM would generally only reveal the external. A sentence has been added into the results with the cell size data as suggested. It is essential for our coccosphere research that all measurements are obtained from the same individual coccosphere to investigate ratios between parameters on a cellular-level and this would not be possible with a mix of LM and SEM work.

40: The authors present calcite production rates in Figure 6. I would appreciate a sentence on how this was calculated in the Methods section i.e. was this an average of all PIC contents x growth rates in exponential phase or just from one specific day? Likewise for the “stationary phase”-values.

Author response: We state the calculation used for PIC production as Eqn. 1 in Sect. 4 and have added a sentence here to specify what data was used for which calculation. In the Fig. 6 caption we do say that (a) that the mean and 25th to 75th percentiles are calculated from exponential days or non-exponential days respectively (data from 22 °C experiment) and (b) shows the full range (minimum to maximum) in PIC, exponential and non-exponential growth rates that we observed respectively across our entire dataset to represent the absolute minimum and maximum values that could occur. Variance in the species-specific shape factor taken from Young and Ziveri (2000) is not included in the PIC calculations.

41: I have found at least 3 errors: de Vargas 2004 and Keller et al. 1987 are missing, whereas Young 2003 is in the citation list, but not cited in the text. Ziveri 2007: check journal name

Author response: de Vargas is in the reference list but under ‘V’ and so has been moved to ‘d’. Keller et al. (1987) has been added, Young (2003) removed, Ziveri (2007) journal name has been changed to the ISI Journal Abbreviation of “Deep-Sea Res. Pt. II”. All references have now been checked for accuracy.

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Response to Reviewer #2

Comments:

Presents novel results, is very well written, has a well reasoned rationale and an overall well structured text, and clear figures.

The study addresses an interesting topic... the data presented are new and of good quality and do support the conclusions drawn by the authors.

In addition, the manuscript is presented in a way that will be also accessible to non-specialists, which is an added value for publication in a multidisciplinary journal such as Biogeosciences.

Specific Comments:

1: Much emphasis is put on the relevance of this study for the investigation/interpretation of coccolithophore geometries in the fossil record as a proxy for coccolithophore growth phase. However, in the introduction the authors state that intact fossil coccospheres can be found in ‘...exceptionally well-preserved sedimentary deposits...’. I would therefore suspect that the application of this growth phase proxy is perhaps useful only in a very limited number of settings and of a few geologic periods where/when intact fossil coccospheres are found. That being the case, the statements about the relevance of this study for the interpretation of the palaeorecords (also in the conclusions) should be toned down, at least in the terms used by the authors. Given that this is not the main reason why this is a valuable piece of work, these statements could be toned down without affecting the relevance and novelty of the study.

Authors response: The presence of coccospheres in sediments is indeed atypical, and our reference to ‘...exceptionally well-preserved sedimentary deposits...’ refers to the very high preservation quality of the calcareous nannoplankton seen in the sediments used by Gibbs et al. (2013) for their study. In other research by the authors (Sheward et al., in prep), ~20 to >300 coccospheres per standard smear slide (see Gibbs et al., 2013 and Bown et al., 2014) have been documented and measured for over 4,000 individual coccospheres of Paleogene age from 11 different sites that range in paleo-latitude between ~58 °S and ~47 °N and from the North Atlantic Ocean, North Pacific Ocean, Indian Ocean and Southern Ocean. Hence, whilst uncommon in large numbers, there is certainly a large range of Paleogene-age sites where intact coccospheres have been found in numbers suitable for robust quantitative analysis. This is likely to be true of other geological periods and sites, but reasoned selection of sections for sampling will be important as hemipelagic sediments (particularly those with less intense bioturbation) are perhaps more likely to contain coccospheres than deep-sea oozes. Several other publications report observations of coccospheres from a variety of locations and ages (e.g., Covington, 1985; Mai et al., 1997; Mai et al., 1998; Mai, 1999). The approach that we present for

the interpretation of coccosphere geometry can also be readily applied to coccospheres found in modern-day plankton samples (see Gibbs et al., 2013), or in surface or Holocene sediments. As these points are not presently expressed in the paper, a statement about applicability is now added into Section 4.3 including describing the present occurrence of fossil coccospheres and we re-worded L 432 – 435 to better state this in the conclusions.

2: Often times in the manuscript it is stated that results are statistically significant, but a section in the methods that specifically presents the statistical approaches used in this study is missing and should be added. Also, data analysis could benefit from some (bootstrap?) outlier analysis, specifically when different properties of the coccolithophore geometry are regressed against one another (e.g., Fig. 2e-h). This would certainly improve the analysis of the high quality (and rich) dataset presented in this study.

Authors response: We agree with Reviewer 2 that a specific methods section presenting our statistical approaches would be beneficial. This methods section now states as follows:

“The relationship of \emptyset with C_L and C_N in each species were tested by Model II reduced major axis (RMA) linear regression analysis and confidence intervals (95 %) for the regression slope were calculated by bootstrapping over 1999 iterations using the freeware Paleontological Statistics (PAST; v. 3.13; Hammer et al., 2001). We compare species-specific mean \emptyset and mean C_N between growth phases using a *t*-test in GraphPad Prism (version 7.0a for Mac OS X; GraphPad Software, Inc., USA). The difference in mean \emptyset or C_N between exponential-phase growth and non-exponential-phase growth were considered significant at $p < 0.05$.”

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Physiology regulates the relationship between coccosphere geometry and growth-phase in coccolithophores

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Abstract. Coccolithophores are an abundant phytoplankton group that exhibit remarkable diversity in their biology, ecology, and calcitic exoskeletons (coccospheres). Their extensive fossil record is testament to their important biogeochemical role and is a valuable archive of biotic responses to environmental change stretching back over 200 million years. However, to realise the full potential of this archive for (paleo-)biology and biogeochemistry requires an understanding of the physiological processes that underpin coccosphere architecture. Using culturing experiments on four modern coccolithophore species (*Calcidiscus leptoporus*, *Calcidiscus quadriperforatus*, *Helicosphaera carteri* and *Coccolithus braarudii*) from three long-lived families, we investigate how coccosphere architecture responds to shifts from exponential (rapid cell division) to stationary (slowed cell division) growth phases as cell physiology reacts to nutrient depletion. These experiments reveal statistical differences in cellcoccosphere size and the number of coccoliths per cell between these two growth phases, specifically that cells in exponential-phase growth are typically smaller with fewer coccoliths, whereas cells experiencing growth-limiting nutrient depletion have larger coccosphere sizes and greater numbers of coccoliths per cell. Although the exact numbers are species-specific, these growth-phase shifts in coccosphere geometry demonstrate that the core physiological responses of cells to nutrient depletion results in increased cellcoccosphere sizes and coccoliths per cell across four different coccolithophore families (Calcidiscaceae, Coccolithaceae, Isochrysidaceae, Helicosphaeraceae), a representative diversity of this phytoplankton group. Building on this, the direct comparison of coccosphere geometries in modern and fossil coccolithophores enables a proxy for growth phase to be developed that can be used to investigate growth responses to environmental change throughout their long evolutionary history. Our data also shows that changes in growth rate and coccoliths per cell associated with growth-phase shifts can substantially alter cellular calcite production. Coccosphere geometry is therefore a valuable tool for accessing growth information in the fossil record, that provides unprecedented insights into the biotic-responses of species to environmental change and it's the potential biogeochemical consequences.

1 Introduction

The fossil remains of biomineralised plankton provide comprehensive records of their biogeography, ecology, diversity and evolution that have significance for our understanding of past ocean and climate systems and its influence on these microscopic organisms. Despite their small size (2 to 200 μm for nanno- and microplankton), the vast numbers of photosynthesising plankton in the ocean drive many regional- to global-scale biogeochemical processes and comprise the biomass that sustains the ocean ecosystem the wider diversity of marine life at higher trophic levels (e.g., Menden-Deuer and Kjørboe, 2016). Investigating the biological response of plankton species to environmental variability is therefore a crucial step in understanding the potential consequences of future climate change on marine systems.

Coccolithophores are a major group of calcifying marine algae that first evolved more than 200 million years ago (Ma) during the Late Triassic (Janofske, 1992; Bown et al., 2004). The remains of their calcite cell coverings contribute to the export of biogenic carbonate to deep-sea sediments (Broecker and Clark, 2009), forming a geographically and temporally extensive fossil record that is mostly in the form of individual calcite plates called coccoliths. Spatial and temporal analysis of coccoliths reveals the evolution, biogeography and ecology of past species (e.g., Haq and Lohmann, 1976; Knappertsbusch, 2000; Ziveri et al., 2004; Gibbs et al., 2006; Baumann et al., 2016) and the response of species and communities to palaeoceanographic and palaeoclimatic variability (e.g., Bollmann et al., 2002; Bown, 2005; Bollmann et al., 2009; Bown and Pearson, 2009).

Valuable new insights into past coccolithophore communities can also be provided by the study of intact fossil coccospheres that have not disarticulated into their component coccoliths, providing intriguing snapshots of individual cell growth in geological time (Gibbs et al., 2013; Bown et al., 2014; O’Dea et al., 2014). Whilst the preservation of intact coccospheres in sediments is generally uncommon, recent investigations showcase a large diversity of coccospheres from a range of ages, ocean basins, and latitudes in numbers suitable for robust quantitative analysis (Gibbs et al., 2013; Bown et al., 2014). The discovery of relatively abundant fossil coccospheres in exceptionally well-preserved sedimentary deposits inspired Gibbs et al. (2013) to first explore the quantitative links between coccosphere geometry (coccosphere size, coccolith length and coccolith number) and population growth. Their laboratory experiments using the modern species *Coccolithus braarudii* and *Emiliana huxleyi* identified that cells undergoing rapid cell division (termed ‘exponential-phase’ growth) were smaller and had fewer coccoliths per coccosphere compared to cells dividing slowly, or not at all (‘stationary-phase’ growth). This initial evidence for a relationship between growth phase and coccosphere geometry was then used to reconstruct the response of fossil taxa (*Coccolithus* and *Toweius*) through an interval of rapid warming ~56 Ma called the Paleocene-Eocene Thermal Maximum (Gibbs et al., 2013; O’Dea et al., 2014). As growth phases describe ‘states’ of rapid or slowed growth rates, these findings hint that coccosphere geometry could provide opportunities for new insights into the ecological ‘fitness’ and subsequent evolutionary success of coccolithophore populations where growth rates (or other physiological measures of fitness) cannot be measured directly.

65 The development of coccosphere geometry as an indicator, or even proxy, of growth phase in the fossil record requires further evidence that phases of rapid and slowed growth produce quantifiably distinct differences in coccosphere geometry, which can be regarded as a ‘universal’ feature of coccolithophores rather than just a species-specific attribute. Even across the diversity of modern species, we observe substantial variability in cell size, coccolith length and numbers of coccoliths per cell. Given this observation, can we reasonably hypothesise that the growth-geometry relationship reported by
70 Gibbs et al. (2013) for two modern species is similar across coccolithophores in general? If this is the case, then coccosphere geometry could prove to be a valuable proxy for growth phase; and ~~hence overall population fitness~~ provide new insights into important fitness-related traits where growth rates cannot be measured directly. One potential concern is that coccolithophores show pronounced species-specific and even strain-specific physiological responses to a variety of environmental manipulations such as carbonate chemistry and nutrient availability in culture experiments (Langer et al.,
75 2006; Langer et al., 2009; Krug et al., 2011), which may extend to coccosphere variability. We therefore require coccosphere geometry data from multiple modern species experiencing different growth phases in order to further investigate the relationship between coccosphere geometry and growth.

Here, we aimed to determine relationships (if any) between growth phase and coccosphere geometry in three modern coccolithophore species - *Calcidiscus leptoporus*, *Calcidiscus quadriperforatus* and *Helicosphaera carteri* - and to integrate
80 these new data with those previously determined by Gibbs et al. (2013) for *Coccolithus* and *Emiliana*. *Calcidiscus* and *Helicosphaera* are particularly pertinent study taxa, as they have widespread modern and geological occurrences and are important components of mid- to low-latitude coccolithophore communities, preferring warmer temperate to tropical waters (Ziveri et al., 2004). These species are also three of the largest and most heavily calcified of all the modern species, along with *Coccolithus pelagicus* in the high latitudes and *Coccolithus braarudii* in the mid- to high-latitudes (Ziveri et al., 2004).
85 ~~and~~ They are therefore important contributors to the production (Daniels et al., 2014; Daniels et al., 2016) and export of inorganic carbon to the deep ocean (Ziveri et al., 2007). Variability in coccosphere geometry in these species, particularly the number of coccoliths per cell, could therefore substantially alter cellular calcite with significant consequences for calcite production and export. The well-documented fossil records of these genera extend back to the first occurrence of *Calcidiscus* ~57 Ma (Bown et al., 2007) and *Helicosphaera* ~54 Ma (Perch-Nielsen, 1985). Alongside *Coccolithus* they have been
90 significant components of coccolithophore communities over much of the last ~55 Ma (Perch-Nielsen, 1985; Bown et al., 2007).

Helicosphaera and *Calcidiscus* also have distinct evolutionary and physiological differences that may highlight restriction of the growth-geometry relationship to specific lineages. Species within the Helicosphaeraceae (Order Zygodiscales) have evolved in a lineage quite separate to the Coccolithaceae and Calcidiscaceae (Order Coccolithales), with
95 the two orders diverging very early in coccolithophore evolutionary history during the Jurassic, ~150-200 Ma (de Vargas et al., 2007). *Helicosphaera carteri* is also physiologically distinct from both *Coccolithus* and *Calcidiscus* species as it is motile in the diploid (heterococcolith-bearing) life-cycle phase. As ~~the genera~~ *Coccolithus* and *Calcidiscus* are considered to be relatively closely related, ~~but are~~ though still classified into separate families, we would predict that the growth-phase

diagnostic features of coccosphere geometry in *Calcidiscus* species might be more likely to show fundamental similarities to those reported for *Coccolithus* by Gibbs et al. (2013). To our knowledge, the experiments undertaken for this study have produced the most extensive dataset of modern coccosphere geometry yet to be presented, comprising a total of more than 13,300 measurements of coccosphere and cell size, coccolith length and coccoliths per cell from 2,850 individual cells.

2 Methods

2.1 Experiment design

Monoclonal cultures of South Atlantic Ocean *Calcidiscus quadriperforatus* strain RCC 1135, *Calcidiscus leptoporus* strain RCC 1130 and *Helicosphaera carteri* strain RCC 1323 were obtained from the Roscoff Culture Collection (RCC) and maintained at an incubation temperature of 19 °C at the National Oceanography Centre, Southampton. Cultures were acclimated to new experimental temperature and light conditions for a minimum of two weeks (>10 generations) prior to the start of each experiment. The light regime remained consistent across all experiments at irradiance levels of 75 to 90 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (equivalent to a daily photon flux of $\sim 73.5 \text{ mol photons m}^{-2} \text{d}^{-1}$) with a 12-hour light, 12-hour dark irradiance cycle. To achieve a range of cell division rates, experiments were undertaken at 16, 18, 20 and 22 °C, which is well within the natural temperature range experienced by field populations of these three species (Ziveri et al., 2004).

For each temperature experiment, all three species were cultured simultaneously and in duplicate following a 'batch culture' procedure, where an initially low number of cells ml^{-1} are left to increase in density, using up nutrients, until initial nutrient levels are completely depleted and population growth ceases. This approach enables coccosphere geometry data to be collected from both nutrient-replete rapid cell-division days and nutrient-deplete slowed cell-division days towards the end of the experiment, as used successfully in the experiments of Gibbs et al. (2013) for *Coccolithus*. The initial starting density of cells for each experiment was $\sim 300 \text{ cells ml}^{-1}$ (taken from acclimated cultures) added to 350 ml of sterilised and filtered natural seawater with added nutrients enriched with 28.8 μM nitrate and 1.8 μM phosphate (lower-nutrient K/20 medium, modified from Keller et al., 1987, following Langer et al., 2006; Gerecht et al., 2014 and Daniels et al., 2014). Lower nutrient media was specifically used to ensure that cultures reached nutrient limiting conditions before the occurrence of significant changes in carbonate chemistry (Daniels et al., 2014). The effect of increasing cell density on the carbonate chemistry of the media over the duration of the experiment was not directly quantified but it is likely that there was DIC consumption throughout the course of each experiment. However, further minimised by our aim was to minimise the effect of cell growth on carbonate chemistry by: using low-nutrient media to ensure that cultures reached nutrient-limiting conditions relatively quickly and at relatively low final cell concentrations; using 650 ml polycarbonate flasks (Thermo Fisher Scientific) with vented lids to allow faster diffusive gas exchange between the culture media and the atmosphere outside the flask; and aerating and mixing each flask daily under sterile conditions to further encourage gas exchange. After initial inoculation of the media, experiment cultures increase in cell number rapidly, termed the exponential growth phase, and were allowed to grow into stationary phase, at which point increasing nutrient limitation reduces growth rates such that

the day-to-day increase in cells ml⁻¹ decreases towards zero. The typical experiment duration between initial inoculation and the onset of stationary phase growth was between 14 and 21 days.

2.2 Growth rate calculation

Daily cell abundance was determined from triplicate counts of cells ml⁻¹ using a Sedgwick Rafter Cell (Pysen-SGI; following Langer et al., 2006) on a transmitted light microscope at x100 magnification. As *H. carteri* is a motile species, 40 µl per ml (4% final volume) 10% formaldehyde was added to the *H. carteri* samples prior to counting to inhibit movement prior to counting to and ensure counting accuracy. Daily growth rates were calculated as the natural log of the difference in cell density between the census day and the day before (Langer et al., 2006). The duration of the exponential growth phase was then determined by visual examination of these daily growth rates and plots of cell abundance over time. Mean exponential growth rates (μ) for each temperature experiment were calculated from daily cell abundances, where $\mu = [\ln(N_t) - \ln(N_0)]/d$, and N_0 and N_t are the cell concentrations at the beginning and end of the exponential phase, respectively, and d is the duration of the exponential phase in days.

2.3 Coccosphere geometry

Samples for light microscope (LM) analysis were taken daily using 2-5 ml of each culture replicate, filtered onto cellulose nitrate filters (pore size 0.8 µm; Sartorius Stedim Biotech) and dried overnight at 50 °C. One half of each filter was then fixed between a glass microscope slide and a cover slip using Norland Optical Adhesive 74 (Norland Products Inc.) and cured under UV light exposure. ~~The other half of each filter was stored for future scanning electron microscope (SEM) analysis or for additional replicate LM slides if needed.~~ All LM analysis was performed using a cross-polarised light microscope (Olympus BX51) with a colour camera attached (Olympus DP71). Coccosphere geometry data was obtained through LM following the same techniques applied by Gibbs et al. (2013) and Daniels et al. (2014), and described in detail here. Random transects across the widest section of the filter hemisphere were performed until 30 individual coccospheres per slide were located from slides corresponding to alternate day or, in some instances, daily samples. First, the number of coccoliths around each cell (C_N) was counted by finely adjusting focal depth. Then, in-focus images of the upper coccosphere surface and maximum cell cross-section were captured from which biometric measurements (Fig. 1) of coccolith length (C_L), coccosphere size (\emptyset ; size including calcite covering) and cell size (\ominus ; size excluding calcite covering and which is assumed to be equivalent to cell diameter) were taken (Cell^D software, Olympus). Unlike the spherical coccospheres of *Coccolithus* and *Calcidiscus* species, *H. carteri* coccospheres are prolate spheroids (Fig. 4), so here we report cell and coccosphere sizes for this species as equivalent spherical diameters. Prolate spheroid volume is calculated as $V = (\pi/6)d^2h$, where d is the short-axis cell/coccosphere diameter and h is the long-axis cell/coccosphere height (Sun and Liu, 2003). This volume is used to calculate equivalent spherical radius. This coccosphere geometry dataset is available from <https://doi.pangaea.de/doi:10.1594/PANGAEA.865403>, ~~doi registration in progress.~~

2.4 Cellular calcite calculation

Particulate inorganic carbon (PIC) per cell was calculated for each individual coccosphere following Young and Ziveri (2000):

$$165 \quad \text{Cellular PIC (pmol C cell}^{-1}\text{)} = \frac{C_N \times C_L^3 \times k_s \times 2.7}{100} \quad (1)$$

where C_N is number of coccoliths per cell, C_L is coccolith length (μm), k_s is a shape factor that numerically describes species-specific coccolith morphology, and $2.7 \text{ pg } \mu\text{m}^{-3}$ is the density of calcite. Division by 100 calculates cellular PIC in pmol C cell^{-1} from pg cell^{-1} . We use the shape factors of $k_s = 0.08$ for *Calcidiscus* spp., $k_s = 0.05$ for *H. carteri*, and $k_s = 0.06$ for *Coccolithus* spp. from Young and Ziveri (2000). Mean, 25th and 75th percentiles, and the range of cellular PIC were
170 calculated from the 22 °C experiment data of each species using coccosphere geometry data from selected mid-exponential-phase days (*C. leptoporus* = days 7, 9, 11; *C. quadriperforatus* = days 3, 5, 7; *H. carteri* = days 6, 7, 8) and all non-exponential-phase days. Mean exponential- and non-exponential-phase calcite production rates at 22 °C were calculated based on these mean cellular calcite values multiplied by mean exponential and non-exponential growth rates, respectively, for the same temperature experiment. The minimum to maximum range in growth rates was based on growth rates observed
175 across all temperature experiments.

2.5 Additional experimental results from *Coccolithus*

This study reports the new experimental results for *Calcidiscus* and *Helicosphaera* alongside coccosphere geometry and growth data for *Coccolithus* from two previous studies that used identical LM methods to collect coccosphere geometry data. Gibbs et al. (2013) obtained coccosphere geometry data from a comparable batch culture experiment at a single temperature
180 in *Coccolithus braarudii* strain RCC 1197. This data is presented for direct comparison with the three new species of this study, as much of the Gibbs et al. (2013) data was originally presented as Supplementary Information to accompany that short-format paper. We also present results from a previously unanalysed dataset of exponential-phase coccosphere geometry in *C. braarudii* strain RCC 1198 and *C. pelagicus* strain RCC 4092, originally published as a data report by Sheward et al. (2014) and available from <http://www.pangaea.de> (doi: 10.1594/PANGAEA.836841). For that study, batch
185 culture experiments were undertaken at multiple temperatures (6-12 °C in *C. pelagicus* and 12-19 °C in *C. braarudii*) and samples for coccosphere geometry analysis collected on a single mid-exponential-phase experiment day (further details in Daniels et al., 2014).

2.6 Statistical analyses

The relationships of \emptyset with C_L and C_N in each species were tested by Model II reduced major axis (RMA) linear regression
190 analysis. Confidence intervals (95 %) for the regression slope were calculated by bootstrapping over 1999 iterations using the freeware Paleontological Statistics (PAST; v. 3.13; Hammer et al., 2001). We compare species-specific mean \emptyset and mean C_N between growth phases using a *t*-test in GraphPad Prism (version 7.0a for Mac OS X; GraphPad Software, Inc.,

USA). The difference in mean \emptyset or C_N between exponential-phase growth and non-exponential-phase growth were considered significant at $p < 0.05$.

195 3. Results

3.1 Growth rates

The four temperature experiments resulted in a modest range of ~~daily and~~ mean exponential growth rates (μ) across *Helicosphaera* and *Calcidiscus* species. The highest ~~mean~~ exponential growth rate for *C. quadriperforatus* was achieved at 22 °C ($\mu = 0.44 \text{ d}^{-1}$), for *C. leptoporus* at 20 °C ($\mu = 0.44 \text{ d}^{-1}$), and for *H. carteri* at 20 °C ($\mu = 0.45 \text{ d}^{-1}$). Mean exponential
200 growth rates for *C. braarudii* at 15 °C were 0.68 d^{-1} . These values are well within the ranges reported in other studies carried out at similar temperatures for *Calcidiscus* (Langer et al., 2006; Buitenhuis et al., 2008; Fiorini et al., 2010; 2011; Langer et al., 2012; Candelier et al., 2013; Müller et al., 2014) and *H. carteri* (Stoll et al., 2002; Šupraha et al., 2015). Exponential growth rates of $0.4\text{-}0.5 \text{ d}^{-1}$ signify that roughly half of the culture population undergoes cell division each day. Maximum cell density was $\sim 100,000 \text{ cells ml}^{-1}$ in *C. leptoporus* cultures, $60\text{-}100,000 \text{ cells ml}^{-1}$ for *C. quadriperforatus*, $\sim 30,000 \text{ cells ml}^{-1}$
205 for *H. carteri* and $\sim 25,000 \text{ cells ml}^{-1}$ for *C. braarudii*.

3.2 Within-species range in coccosphere geometry

Coccosphere (\emptyset) and cell size (Θ), coccolith length (C_L) and number of coccoliths per cell (C_N) show clear species-specific differences (Fig. 2, Table 1). A considerable range in \emptyset is seen in all species; ~~15~~13.8 ~~µm~~ to ~~25~~24.4 µm in *C. quadriperforatus*, ~~and~~ 109.4 ~~µm~~ to 1520.9 µm in *H. carteri* and 10.0 to 19.7 µm in *C. leptoporus*. This is a comparable \emptyset
210 range to *C. pelagicus* (~~12~~11.7 to ~~22~~20.8 µm) but slightly less than the \emptyset range observed in *C. braarudii* (~~15~~13.7 to ~~30~~29.7 µm). Cell size exhibited a similarly large range of 6.5 to 18.0 µm in *H. carteri*, 6.4 to 16.5 µm in *C. leptoporus*, 8.6 to 18.8 µm in *C. quadriperforatus*, 7.9 to 18.1 µm in *C. pelagicus*, and 9.9 to 15.8 µm in *C. braarudii* (Table 1).

Calcidiscus spp. and *H. carteri* show a much greater range in C_N compared to *Coccolithus* spp. (Fig. 2e-h). The most frequently observed C_N is 16 in *H. carteri* cells, 18 in *C. quadriperforatus* cells, and 19 in *C. leptoporus* cells, with a
215 maximum number of ~ 30 coccoliths in all of these species. In one *C. leptoporus* cell, the coccosphere was formed from 45 coccoliths (Fig. 4c). In contrast, *Coccolithus* cells more typically have 11 to ~~12~~14 coccoliths per cell, up to a maximum of 20-~~23~~ coccoliths. ~~In one C. leptoporus cell, the coccosphere was formed from 45 coccoliths (Fig. 4e).~~ The relationship between C_N and \emptyset subsequently shows a steeper gradient in *Helicosphaera* and *Calcidiscus* (greater C_N increase per µm \emptyset) compared to *Coccolithus* (Fig. 2). The ~~comparable similar~~ coccosphere sizes but significantly greater number of coccoliths
220 per coccosphere of *C. quadriperforatus* compared to *C. braarudii*, and *C. leptoporus* compared to *C. pelagicus*, ~~suggests indicates~~ that *Calcidiscus* species achieve a greater degree of coccolith overlapping compared with *Coccolithus* species of a similar coccolith size. This is likely the result of the circular shape and narrower central tube structure in *Calcidiscus* coccoliths, which therefore pack more tightly around the cell with increasing C_N , moderating a corresponding increase in \emptyset .

The minimum C_N in *H. carteri* is similar to *Coccolithus* ($C_N = 6$ and $C_N = 5-7$, respectively). The smallest *H. carteri* cells, with just 6 coccoliths, ~~observed in *H. carteri*~~, formed cuboid coccospheres (Fig. 4a) and are most likely recently-divided cells. Cubiform coccospheres have also been reported in Bown et al. (2014) for the extinct Paleogene taxa *Toweius pertusus* and *Umbilicosphaera bramlettei* and ‘boxy’ coccospheres are also seen in several *Chiasmolithus* species, which are probably also related to small cell sizes soon after cell division.

Although coccosphere geometry is similar in the two ~~closely-related~~ *Calcidiscus* species (Fig. 2f, g), it is not identical, with *C. leptoporus* producing coccospheres with a slightly greater C_N on average than *C. quadriperforatus* (slopes of 3.01 and 2.11, respectively). In contrast, the two species of *Coccolithus* are more closely comparable, with the linear regression gradient between \emptyset and C_N ~~the same~~ 1.50 and 1.35 in ~~both~~ *C. pelagicus* and *C. braarudii*, although the gradients are offset from each other (y-intercepts of -10.17 and -17.33, respectively; Fig. 2h). Until recently, these two *Calcidiscus* species were considered to be intraspecific morphotypes (Knappertsbusch et al., 1997; Knappertsbusch, 2000) or sub-species (Geisen et al., 2002) but have since been shown to be genetically-distinct, which is also the case for *C. pelagicus* and *C. braarudii* (Sáez et al., 2003; de Vargas et al., 2004). The considerable overlap in C_L , \emptyset and C_N in *Calcidiscus* species makes species-differentiation based solely on any one of these parameters difficult. However, the species-specific coccosphere geometry identified here lends further support to the genetic distinction between these species, alongside previously identified morphological and ecological differences (Knappertsbusch et al., 1997; Knappertsbusch, 2000; Geisen et al., 2002; Renaud et al., 2002; Sáez et al., 2003; Geisen et al., 2004; Baumann et al., 2016).

Coccolith length varies between cells by up to 4.5 μm in *H. carteri*, 6.0 μm in *C. quadriperforatus*, and 3.7 μm in *C. leptoporus*, which is similar to C_L ranges ~~between~~ of 3.0 to 8.5 μm reported in selected studies on sediment samples (e.g., Baumann, 2004; Henderiks and Törner, 2006; Herrmann et al., 2012; Baumann et al., 2016). Unfortunately, no culturing experiments on *Calcidiscus* or *Helicosphaera* report C_L measurements for comparison. In contrast to C_N , C_L shows no relationship with \emptyset within these clonal populations (Fig. 2i-l) and superimposing C_N onto plots of \emptyset against C_L (Fig. 2m-p) clearly demonstrates the strong co-variance of \emptyset and C_N . In our clonal populations, ~~cell division is fully synchronised across cells resulting in cells have~~ relatively restricted ranges in \emptyset and C_L that have no statistically-significant relationship (Fig. 2i-l). A weak relationship between \emptyset and C_L appears to exist in *Coccolithus* when data for *C. pelagicus* is combined with data from two strains of *C. braarudii* (Fig. 2l, p). This C_L - \emptyset relationship only occurs in these culture experiments when data from several growth-synchronised populations are mixed. ~~an~~ This effect ~~illustrated~~ is also seen in the culture and field data of Gibbs et al. (2013) and ~~that~~ is greatly amplified in fossil assemblages, which ~~that~~ typically integrate the remains of surface populations over longer time-spans (Gibbs et al., 2013, their Fig. 3a). In our single-clone culture populations, however, the principle coccosphere geometry relationship is between C_N and \emptyset rather than C_L and \emptyset .

3.3 Coccosphere geometry as a function of growth

This study demonstrates that coccosphere size in all the species studied is statistically smaller during ~~experiment~~ days of rapid, nutrient-replete, exponential-phase growth than during days of slowed, nutrient-depleted, ~~early-stationarynon-~~

exponential-phase growth (Fig. 3). Mean \emptyset across all four temperature experiments during exponential-phase growth is 14.8 μm in *H. carteri*, 18.4 μm in *C. quadriperforatus*, 13.1 μm in *C. leptoporus* and 20.5 μm in *C. braarudii*. Mean Coccosphere diameter during non-exponential growth is modestly but statistically (unpaired t-test) larger than during exponential-phase growth, with mean \emptyset 0.55 μm larger in *C. quadriperforatus* ($t=3.324$, $df=839$, $p<0.001$), and 0.64 μm larger in *H. carteri* ($t=4.659$, $df=990$, $p<0.0001$), and 0.70.90 μm larger in *C. leptoporus* ($t=5.669$, $df=1020$, $p<0.0001$). Mean \emptyset in *C. braarudii* (Gibbs et al., 2013) shows a larger increase of 1.751.34 μm ($t=9.216$, $df=548$, $p<0.0001$) between exponential- and early-stationarynon-exponential-phase growth. An increase in cell size has also previously been observed in response to nutrient limitation in *Coccolithus* and *Helicosphaera* (Gerecht et al., 2014; Gerecht et al., 2015; Šupraha et al., 2015).

In addition to size differences, coccospheres also typically consist of fewer coccoliths during exponential-phase growth and a greater number of coccoliths during early-stationarynon-exponential-phase growth (Fig. 3). This is shown by an increased frequency of cells in higher C_N classes and an increased mean C_N during non-exponential phase growth in each species. Cells no longer able to maintain exponential rates of growth have an average of 1 to 2 extra coccoliths per cell in *H. carteri* ($t=5.067$, $df=990$, $p<0.0001$) and *C. quadriperforatus* ($t=5.451$, $df=840$, $p<0.0001$), 2 to 3 extra coccoliths per cell in *C. leptoporus* ($t=6.312$, $df=1020$, $p<0.0001$) and 3 to 4 extra coccoliths per cell in *C. braarudii* ($t=14.24$, $df=548$, $p<0.0001$). The frequency distribution of C_N for each species (Fig. 3) can be used as a quantitative indicator of whether cells are in a recently-divided state (close to the minimum number of coccoliths per cell observed, $C_N \leq 10^{\text{th}}$ percentile of the data) or are in a ready-to-divide state (close to the maximum number of coccoliths per cell observed, $C_N \geq 90^{\text{th}}$ percentile of the data). These C_N ‘thresholds’ for recently-divided and ready-to-divide cells for each species are shown in Fig. 3 and Table 1. Based on the species-specific geometries observed, recently-divided cells typically have $C_N \leq 12$ in *H. carteri* and $C_N \leq 14$ in *Calcidiscus* spp., whilst cells that are ready to divide have $C_N \geq 21$ in *H. carteri*, $C_N \geq 23$ in *C. quadriperforatus*, and $C_N \geq 25$ in *C. leptoporus* (Fig. 3). During exponential growth, the mean and frequency distribution of population C_N of the population is typically skewed towards the minimum observed C_N and therefore the population has a higher percentage of ‘recently-divided’ coccosphere geometries, whereas Ppopulations exhibiting slowed growth are more likely to have a greater number an increased percentage of cells in a ‘ready-to-divide’ state. However, there are always some ‘recently-divided’ cells and some ‘ready-to-divide’ cells in both exponential- and non-exponential-phase populations due to on-going cell division, albeit at different rates. There is therefore a large overlap in \emptyset and C_N size-range between exponential- and non-exponential-phase populations (Fig. 3), with negligible change in the maximum \emptyset and C_N of each (Table S1).

285 3.4 Cellular particulate inorganic carbon

PIC can be calculated directly from the extensive dataset of coccosphere geometry collated for this study by multiplying C_N by individual coccolith calcite, following Eq. (1) (Sect. 2.4; Young and Ziveri, 2000). Mean exponential-phase cellular PIC (calculated at mid-exponential-phase for each temperature experiment) was 10.7 to 12.6 pmol C cell⁻¹ in *C. leptoporus*, 21.3 to 25.8 pmol C cell⁻¹ in *H. carteri*, but higher in *C. quadriperforatus* (21.5-30.0 pmol C cell⁻¹) and *C. braarudii* (27.9 pmol

290 C cell⁻¹) (Table 1). At 22 °C, mean PIC during non-exponential experiment days was 9 to 45 % higher compared to mid-
exponential-phase across all species due to an increase in median C_N of 2 to 4 coccoliths (Table S1). The 25th and 75th
percentiles are also clearly shifted towards higher cellular PIC in cells no longer growing exponentially (Fig. 5a.). The 25th
percentile increases 50 to 60% in *Calcidiscus*, with *C. braarudii* and *H. carteri* increasing by 20 to 25%. The increase in 75th
percentile is not as large, but is still considerable in *C. leptoporus* and *C. braarudii* at 36% and 24%, respectively, with *C.*
295 *quadriperforatus* and *H. carteri* showing more modest increases of 6% and 11%.

4. Discussion

4.1 Physiological insights into coccosphere geometry

Within these experiments, coccosphere size (\emptyset) and the number of coccoliths per cell (C_N) varied depending on whether the
culture population was increasing in cell numbers each day at a rapid rate (exponential-growth phase) or a slowed rate (non-
300 exponential-growth phase). Across all four species investigated, the transition from exponential into non-exponential phase
growth was clearly associated with a shift towards ~~an increased abundance of~~ cells with a greater C_N (mean C_N increased by
1-3 coccoliths per cell) and larger coccosphere sizes ~~by 2 μm~~ (mean \emptyset increased by 0.6 μm in *H. carteri* and *C.*
quadriperforatus, 0.9 μm in *C. leptoporus* and 1.3 μm in *C. braarudii*; Fig. 3). ~~(Fig. 3)~~; This represents a statistically
significant increase of ~~10-15%~~ 4 to 7% on exponential-phase ~~size~~ mean \emptyset and an increase of 10 to 27% on exponential-phase
305 mean C_N (t -test, $p < 0.0001$). C_N is not a frequently recorded variable but where \emptyset and C_N in both nutrient-replete and nutrient-
deplete cultures can be inferred from supplementary information (Balch et al., 1993; Paasche, 1998; Gerecht et al., 2014;
Gerecht et al., 2015; Šupraha et al., 2015) these are consistent with the extensive observations from our experiments for
Calcidiscus and *H. carteri* and ~~those of~~ ~~in~~ Gibbs et al. (2013) for *C. braarudii*.

The relationship between growth phase, \emptyset and C_N can be understood by considering the process of cell division and
310 how it is affected by the nutrient depletion that instigates non-exponential-phase growth. Both \emptyset and C_N vary as each cell
progresses through the cell division cycle (unpublished observations; Taylor et al., 2007; Müller et al., 2008). ~~Cells that have~~
~~recently undergone division~~ Recently-divided cells are small with approximately the minimum number of coccoliths
required to form a complete cell covering (unpublished observations; Fig. 4). After division, cells recommence coccolith
production, ~~which is shown in these experiments to~~ and increase C_N until the cell has sufficient coccoliths to cover two
315 newly divided cells. Coccosphere diameter correspondingly increases alongside increasing C_N as the cell synthesises organic
cellular components such as proteins, lipids and carbohydrates. Cultures that are able to maintain exponential rates of cell
division subsequently have a lower mean \emptyset , \emptyset and C_N as the majority of cells are in a ‘recently divided’ state (Fig. 3, 4).
When cells are no longer able to maintain exponential rates of cell division, in this instance due to decreasing nutrient
availability, they divide less frequently on average. This is observed in the later days of each experiment as an increase in the
320 mean \emptyset , \emptyset and C_N , an interpretation that is consistent with the findings of Gibbs et al. (2013).

An increase in cell size, Θ , under decreasing nutrient availability may seem counterintuitive, as nutrients are essential for phytoplankton growth. Nitrate and phosphate are the two key nutrients required by most phytoplankton (Arrigo, 2005; Moore et al., 2013) and they fulfil different purposes within the cell. Phosphate limitation primarily impedes production of the RNA, phospholipids and DNA that are essential for cell replication, and phosphate is a key component of cellular energy carriers (Zhao et al., 2015). Nitrate limitation particularly impacts the synthesis of proteins and pigments used in photosynthesis (Zhao et al., 2015). ~~Nutrient limitation therefore suppresses cell division and growth in multiple ways.~~ However, despite the suppression of cell division and photosynthetic activity by phosphate and nitrate limitation, respectively, the cell is still able to synthesise non-essential lipids and carbohydrates. Cell size and particulate organic carbon content (POC) are therefore able to ~~have been consistently shown to~~ increase under nutrient limited conditions (e.g., Müller et al., 2008) ~~as the cell is still able to produce non-essential lipids and carbohydrates.~~ A similar increase in POC could also reflect DIC limitation, which sometimes results from DIC drawdown as cell numbers rise to high concentrations in non-exponential-phase growth. An increase in POC under DIC limitation was previously shown in *C. braarudii* (Rickaby et al., 2010).

The greater C_N of coccospheres during non-exponential-phase growth (Fig. 3) includes the occurrence of some large coccospheres with very high C_N (Fig. 4) and more than enough coccoliths to cover two daughter cells. This is evidence that cellular calcification (coccolith production) can proceed uninterrupted despite decreasing nutrient availability and indicates that the calcification process has a lower nutrient ‘cost’ compared to cell division processes (Paasche, 1998; Monteiro et al., 2016). This is also illustrated by the dramatic overproduction of coccoliths in *E. huxleyi* under nutrient limitation (Balch et al., 1993; Paasche, 1998), and supported by the C_N evidence from *Calcidiscus* and *Helicosphaera* in this study and *Coccolithus* in Gibbs et al., (2013). An alternative possibility is that the continued production of coccoliths by cells in stationary phase leaves them poised and ‘ready-to-divide’ should nutrients become newly available. In support of this, the recommencement of cell division in stationary phase cultures after the addition of nutrient-replete seawater has been observed in *E. huxleyi* cultures (J. Young, pers. comm.).

4.2 Contrasting growth phase and growth rate

The clear relationship we observe between growth phase, \emptyset and C_N is interpreted to be the result of cellular physiology (calcification, biomass production and the synthesis of molecules involved in cell division) responding to shifts in nutrient availability over the course of the experiments, with stationary-phase nutrient depletion decreasing growth rates to zero once levels became inhibiting to cell division. Exponential-phase growth rates, the proportion of the culture undergoing cell division between two consecutive days (daily growth rates) or averaged across multiple days (mean exponential growth rates), are instead affected by temperature; (which determines the rate of nutrient uptake and the rate of metabolic cell processes); and irradiance; (which affects photosynthetic rates, i.e., the rate at which the cell can produce energy). Our manipulation of experiment temperature (16-22 °C) aimed to achieve a range of exponential-phase growth rates that might reveal any correlation between growth rate and coccosphere geometry. However, no clear relationship between \emptyset , Θ , C_L or

C_N and exponential growth rate (daily or mean) was observed in our experiments. One explanation for this might be that mean exponential growth rates (μ_{exp}) were not that sensitive enough to the temperature range we applied (*C. quadriperforatus* $\mu_{\text{exp}}=0.30-0.44$ d⁻¹; *C. leptoporus* $\mu_{\text{exp}}=0.36-0.31-0.44$ d⁻¹; *H. carteri* $\mu_{\text{exp}}=0.29-0.44-0.28-0.45$ d⁻¹). In addition, growth rates would not necessarily be expected to influence coccosphere geometry in the same way as a shift in growth phase caused by nutrient depletion, as temperature and light primarily affect physiological rates (e.g., Eppley, 1972; Falkowski et al., 1985), whilst nutrient limitation primarily impedes molecule synthesis (e.g., Zhao et al., 2015). Calcification, for example, is contingent on both the rate at which nutrients can be supplied to the cell (temperature-dependent) and processed into energy (light-dependent) and can proceed under nutrient limitation, as shown in our experiments, but may be less efficient under sub-optimal temperature or light conditions. As yet, no studies have investigated the response of cell size and/or coccosphere geometry under a range of optimum vs. limiting temperature or light conditions in coccolithophores. As growth phase describes two different physiological states, one of which manifests as slowed to zero daily growth rates caused by depleted nutrient availability, the ability to identify coccolithophore populations with coccosphere geometries characteristic of each growth phase is an important advance in interpreting growth information directly from the coccosphere. Valuable additional perspectives on the specific role of growth rate on coccosphere geometry would be gained from future work using semi-continuous or continuous culturing techniques to achieve a range of steady-state exponential growth rates under different nutrient, temperature or light conditions.

4.3 Coccosphere geometry as a proxy for growth phase in the fossil record

A notable finding of this study is that coccosphere geometry (coccosphere size, coccolith length and coccoliths per cell) is species-specific but \emptyset and C_N responds identically to growth phase changes across four different species of *Calcidiscus*, *Coccolithus* and *Helicosphaera*. This strongly suggests that coccosphere geometry within the major coccolithophore families Calcidiscaceae and Helicosphaeraceae responds to nutrient-driven changes in growth phase, and therefore cell physiology, in the same way as species within the families Coccolithaceae (Gibbs et al., 2013) and Noelaerhabdaceae (Balch et al., 1993; Paasche, 1998; Gibbs et al., 2013). This is compelling evidence that, as a group, coccolithophores express a common physiological response to shifts from exponential to non-exponential (stationary) growth phase, that is seen as a modest but significant increase in the average C_N and \emptyset of a population (Fig. 3, 4). This specifically results from the ability of the cell to maintain calcification processes even when rates of cell division are suppressed by nutrient limitation.

One of the aims of this study was to further develop the proxy application of fossil coccosphere geometry first that was initially proposed by Gibbs et al. (2013) for *Coccolithus* and *Toweius*. Culture experiments on *Coccolithus* and *Emiliana huxleyi* showed that \emptyset and C_N responded to growth phase as described above and they Gibbs et al. (2013) applied this as a framework for interpreting the to coccosphere geometry records of fossil *Coccolithus* and an ancestor of *E. huxleyi* called *Toweius* (an ancestor of *E. huxleyi*) across during the Paleocene-Eocene Thermal Maximum climate change event (56 Ma). Given the tendency of coccolithophores to show strong species- and strain-specific responses to external parameters factors, applying this framework extending this application to other fossil species would be might be seen as

highly speculative based on data from only two modern species. The new experimental data presented here for *Calcidiscus* and *Helicosphaera*, in combination with previous results from *Coccolithus* and *Emiliana* (Balch et al., 1993; Paasche, 1998; Gibbs et al., 2013; Gerech et al., 2014; Gerech et al., 2015), provides validity that coccosphere geometry persistently responds to growth phase in a common manner, regardless of species, and notably that mean population C_N increases under slowed growth in *Calcidiscus*, *Helicosphaera*, *Coccolithus* and *Emiliana*.

To further develop this proxy we need to establish threshold values of C_N that identify recently-divided cells and cells with theoretically sufficient coccoliths to undergo cell division. As an exponential-growth phase population is undergoing cell division at a rapid rate, it has a greater percentage of recently-divided cells ($C_N \leq$ lower threshold: Table 1). In contrast, a slowly-dividing population in stationary-phase growth has a greater percentage of ready-to-divide cells ($C_N \geq$ upper threshold; Table 1) but fewer recently-divided cells. Here, we report these C_N threshold values (Table 1) for *Calcidiscus* and *Helicosphaera* (Fig. 3) and add them to those identified by Gibbs et al. (2013) for *Coccolithus*. C_N is relatively easy to measure in both fossil and modern coccospheres using light microscopy, and so potentially provides a robust method for identifying populations that are growing rapidly (exponential populations where $> \sim 15\%$ population is characterised by cells with C_N typical of recently-divided cells) compared to populations that are growing slowly (non-exponential populations where $> \sim 15\%$ population is characterised by cells with C_N typical of ready-to-divide cells). This is illustrated in Fig. 56 and these specific C_N threshold values can be used to approximate the growth state of any fossil or modern population of *Coccolithus*, *Helicosphaera* or *Calcidiscus* species. In reality, the mixing of populations of different growth states in the fossil record (and open ocean) will frequently result in percentages of recently-divided and ready-to-divide cells for a population frequently lying between the two end-members shown in Fig. 56 (Gibbs et al., 2013). However, where time-series of coccosphere geometry data are available, intervals of changing growth states can be identified as substantial temporal shifts in the proportional percentage of recently-divided to ready-to-divide cells indicative of less or more favourable growth conditions (Gibbs et al., 2013; O’Dea et al., 2014). Whilst in these experiments non-exponential growth phase is initiated by nutrient depletion, this would be an overly simplistic interpretation for modern field, sediment trap or fossil populations. It is more reasonable to interpret shifts in population coccosphere geometry as a response to less or more favourable growth environments, incorporating a combination of nutrient, temperature, light and other environmental factors that may influence population growth.

For fossil taxa that have no direct modern counterpart, the general characteristics of rapidly growing populations consisting of an increased proportion of smaller cells with fewer coccoliths relative to slowly dividing populations can be used as a qualitative indicator of changes in growth-phase through time. Based on the species studied here, the typical C_N of recently-divided and ready-to-divide cells can be tentatively proposed for any species (living or extinct) based on the 10th and 90th percentiles of the C_N histogram produced from a compiled taxon-specific dataset of coccosphere geometry. Relative changes in the distribution of C_N through time within any species can then provide a valuable indication of intervals where species may be experiencing nutrient conditions that are more (shift towards lower C_N , recently-divided geometry) or less (shift towards higher C_N , ready-to-divide geometry) favourable for growth. This can be achieved for any taxon by first

compiling a dataset of coccosphere geometry for the focal species and then calculating the 10th and 90th percentiles to estimate taxon-specific C_N thresholds. Growth phase can then be estimated by calculating the percentage of each sample with coccospheres of ready-to-divide and recently-divided C_N before plotting as Fig. 56. We would caution users to be mindful that the full range of coccosphere sizes in a species may not be represented in any particular sample and that, as fossil species are typically morphospecies concepts, the range in C_N observed is likely to incorporate multiple intraspecies morphotypes or ecotypes with similar but subtly varied coccosphere geometries. We therefore recommend that as many samples as is feasible are considered in the full dataset before calculating C_N thresholds and that the minimum to maximum range in coccosphere geometry parameters (Table 1) within modern species are heeded as an indication of how variable coccosphere geometry can be within even a single clone. **The coccolithophore fossil record is also vulnerable to size-related preservational biases (Young et al., 2005) that may affect the abundance of very small or very large coccospheres within a sample and may not be consistent through time at the same site. The overall quality of preservation should therefore be considered when interpreting changes in coccosphere geometry through time, and caution should be exercised if there is a suspected strong bias against the preservation of particular taxa, very small coccoliths or very small or very large coccospheres within a sample.**

Thus far, relatively common fossil coccospheres have been documented from at least 24 localities (Burns, 1975; Covington, 1985; Lambert, 1987; Young and Bown, 1991; Mai, 1997; Mai et al., 1998; Henderiks, 2008; Ciurej, 2010; Bown et al., 2014) representing low to high latitudes, the North and South Atlantic Oceans, North Pacific Ocean, Indian Ocean and Southern Ocean, and ranging from Kimmeridgian (Late Jurassic) to Pleistocene. Coccosphere geometry analysis is therefore likely to prove applicable at a range of localities and time intervals, but reasoned selection of sampling sections is likely to be important for retrieving sufficient coccospheres for robust data analysis. Hemipelagic sediments, particularly those with less intense bioturbation, are perhaps more likely to contain coccospheres than deep-sea oozes (Bown et al., 2014).

Whilst we conclude that coccosphere geometry can be used with confidence as a proxy of growth phase in the fossil record or modern ocean, we must be clear that the environmental and growth signal recorded in field populations is always more complex than any laboratory experiment results. Populations may only experience a specific nutrient state for a few weeks or less before conditions change and the coccosphere geometry response of any individual cell ~~to the experienced nutrient state~~ is likely to be further complicated by temperature and light conditions that are also essential for growth. At present there is little to no experimental data to demonstrate the response of coccosphere geometry ~~specifically~~ to temperature or irradiance, or how changes in growth rate specifically (rather than growth phase) ~~driven by these conditions~~ may manifest in coccosphere geometry. The fossil record of ~~eococcosphere geometry~~ coccolithophores further compounds these considerations, as fossil assemblages are typically temporal integrations of many thousands of very short-lived population states. The coccosphere geometry signal of species populations transitioning between rapid and slowed growth phases ~~as nutrient conditions change through time~~ clearly becomes obscured and diluted by the mixing of population remains and subtle shifts in species morphotypes and ecotypes as environmental conditions vary, as illustrated by Gibbs et al. (2013).

455 Nevertheless, the use of coccosphere geometry as a proxy for growth phase is valid across coccolithophores generally, and not just specific species. As exponential- and stationary-growth phases describe two distinct physiological states, the former with rapid growth rates experiencing optimal nutrient supply and the latter with slowed growth rates suffering nutrient limitation, coccosphere geometry provides a unique link ~~between~~ to the physiology of individual cells, ~~and may contribute towards our understanding of~~ population fitness (measured as growth rate ~~or fitness-related traits such as cell~~ size and calcification) and ultimately the long-term success of species responding to varying nutrient conditions. As such, the coccosphere geometry-growth phase proxy is a highly valuable tool, for ~~the first time allowing direct considerations of~~ growth phase in evolutionary and palaeoceanographic studies.

4.4 Implications of growth-driven cellular PIC and POC for calcite production

Coccolithus, *Calcidiscus* and *Helicosphaera* are potentially major regional calcite producers in both the modern (Daniels et al., 2014; Daniels et al., 2016) and past ocean (Ziveri et al., 2007), as they are some of the largest, most heavily calcified modern species with distributions throughout sub-Polar (*C. pelagicus*), temperate (*C. braarudii*), and sub-tropical (*Calcidiscus* and *Helicosphaera*) oceans (Ziveri et al., 2004). The process of biogenic calcification is thought to be responsive to climate and particularly sensitive to changes in ocean carbonate chemistry (for reviews see Riebesell and Tortell, 2011; Bach et al., 2015; Meyer and Riebesell, 2015). Our experiments show that calcite per cell can ~~also~~ change significantly with growth phase ~~by means of variability in~~ as the number of coccoliths in the coccosphere ~~varies in response as cellular physiology responds~~ to changing nutrient availability. This was not ~~previously~~ known for any species other than *E. huxleyi*, which produces high C_N , multi-layered coccospheres under nutrient limitation. *E. huxleyi* additionally sheds excess coccoliths into the surrounding waters (e.g., Balch et al., 1993), potentially amplifying the biogeochemical impact of increased coccolith production under low nutrient conditions, although, to our knowledge, this species is unique in this respect. Calcite production is a function of cellular calcite (particulate inorganic carbon, PIC) and growth rate, and could therefore change considerably with environmental conditions through time with implications for the biogeochemical cycling of carbon in the ocean.

PIC can be calculated directly from ~~the extensive dataset of~~ coccosphere geometry ~~collated for this study. This is achieved~~ by multiplying C_N by individual coccolith calcite, following Eq. (1) (Sect. 2.4; Young and Ziveri, 2000). Using the 22 °C experiment as an example, mean exponential-phase cellular calcite ranged from 10.91 pmol C cell⁻¹ in *C. leptoporus* to 19.3 pmol C cell⁻¹ in *C. quadriperforatus*. In the non-exponential-phase, cellular calcite increased by 4.9 pmol C cell⁻¹ (45%) in *C. leptoporus*, 2.6 pmol C cell⁻¹ (9%) in *C. quadriperforatus*, 2.9 pmol C cell⁻¹ (11%) in *H. carteri* and 5.9 pmol C cell⁻¹ (21%) in *C. braarudii* (Fig. 5; Table S1) due to the higher C_N proportion of each population with greater C_N (Fig. 3). ~~Mean cellular PIC averaged across exponential growth phase days in the 22 °C experiment is 11–15 pmol C cell⁻¹ in *C. leptoporus* and 16–18 pmol C cell⁻¹ in *H. carteri*, but higher in *C. quadriperforatus* and *C. braarudii* at 30 pmol C cell⁻¹ (Fig. 6). Mean PIC increases during non-exponential experiment days (22 °C) in all species due to an average increase of 2–4 coccoliths per cell (Fig. 3). The 25th and 75th percentiles are also clearly shifted towards higher cellular PIC in cells no longer growing~~

490 exponentially (Fig. 6a). *Calcidiscus* 25th percentile increases 50-60%, with *C. braarudii* and *H. carteri* increasing 20-25%.
The increase in 75th percentile is not as large but is still considerable in *C. leptoporus* and *C. braarudii* at 36% and 24%,
respectively, with *C. quadriperforatus* and *H. carteri* showing more modest increases of 6% and 11%. Calcite production per
cell per day ($\text{pmol C cell}^{-1} \text{d}^{-1}$) is can be calculated by multiplying cellular calcite (pmol C cell^{-1}) by growth rate (d^{-1}) (e.g.,
Daniels et al., 2014; 2016). Calcite production in these four species is 6 to 20 times higher than in *E. huxleyi* at a comparable
growth rate (Fig. 65; Balch et al., 1996; Poulton et al., 2010), therefore and hence these heavily calcified species (e.g., the
calcite of one *C. braarudii* cell is equivalent to ~78 cells of *E. huxleyi*) do not necessarily need to be abundant or maintain
495 comparative growth rates to still dominate calcite production (Daniels et al., 2014; Daniels et al., 2016). A dramatic
difference in calcite production can be seen between populations growing exponentially and those no longer growing
exponentially, with reductions in calcite production of 77 to 88% in all species due to the approximate order of magnitude
decrease in growth rates (based on mean exponential and non-exponential growth rates for the 22 °C experiment; Fig. 65c).
In field populations, growth rates can reach as low as $<0.2 \text{d}^{-1}$ (Poulton et al., 2014), similar to the culture populations in
500 slowed growth shown in Fig. 65, and therefore these shifts to such low calcite production per cell per day are approximate
minimum calcite production values for these species. However, it is clear that rates of calcite production can be altered by up
to 50% for even a moderate change of growth rate of 0.1 to 0.2d^{-1} , for example where coccolithophore populations
experience changes in nutrient supply, temperature or light availability that no longer support optimal rates of cell division
(Poulton et al., 2010; 2014).

505 The majority of studies attribute the response of environmentally-driven changes in calcite production to
environmental change to changes variation in calcite per coccolith, through coccolith size, thickness or malformation (e.g.,
Beaufort et al., 2011; Horigome et al., 2014). However, C_L would need to increase by roughly 5 to 20% to achieve the same
change in cellular calcite as that produced by the an increase of just 2 to 4 coccoliths per cell based on our data. O’Dea et al.
(2014) similarly found that changes in coccolith calcite mass of ~5 to 11% and ~6 to 16% for *Toweius pertusus* and
510 *Coccolithus pelagicus* during the PETM were dwarfed by up to 500% changes in cellular calcite resulting from combined
changes in C_L , \emptyset , and C_N across the same time interval of Paleogene climate change. Changes in C_N with growth phase are is
therefore key when considering the impact of environmental parameters such as nutrient availability on cellular PIC and
calcite production rates. The dominant control of growth rates on calcite production, as demonstrated recently by Gerecht et
al. (2015) for *C. pelagicus*, is an important consideration that is often overlooked when investigating the impact of climate
515 on long-term calcite production, carbon export, and sequestration and should be accounted for alongside growth phase
changes in calcite.

5. Conclusions

Experiments on modern species of the coccolithophores *Calcidiscus* and *Helicosphaera* have shown significant differences
in coccosphere geometry under exponential-phase growth (nutrient replete conditions) and non-exponential-phase growth

520 (nutrient depleted conditions) identical to those previously observed in *Coccolithus* and *Emiliania huxleyi*. The extension of
these ~~previously published~~earlier findings into two additional families demonstrates that the decoupling of cell division and
calcification rates in coccolithophores is a core physiological response to nutrient depletion revealed and is expressed in
coccosphere geometry as an increase in coccoliths per cell and coccosphere size. With due consideration, coccosphere
geometry can now be applied as a proxy for growth phase in the geological record, as well as in sediment trap and modern
525 field population samples, with the expectation that populations of any coccolithophore species experiencing growth-limiting
nutrient conditions will have a greater number of larger cells with more coccoliths per cell. The variability of coccosphere
geometry with growth, specifically calcite production through the production of coccoliths, identifies coccoliths per cell as
an equally important parameter as calcite per coccolith in determining cellular calcite. Growth rate is the principal driver of
calcite production rather than cellular calcite, highlighting the need for accessing consideration of growth information in both
530 the modern ocean and geological record in order to explore the impact of future climate change scenarios on calcite
production and export.

Data availability

The coccosphere geometry data and accompanying culture conditions generated for this study are publically accessible as
Sheward et al. (2016) at <https://doi.pangaea.de/10.1594/PANGAEA.865403>, ~~doi registration in progress~~.

535 **Author contributions**

RS, AP and SG conceived the design of the experiment with advice from CD. RS performed the culturing experiments,
collected and analysed the data. RS, SG and AP interpreted the findings. RS wrote the manuscript with contributions from all
authors.

540 The authors declare that they have no conflict of interest.

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Tables

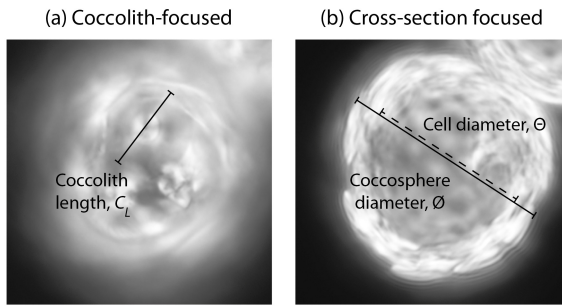
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Table 1. Summary statistics of species-specific coccosphere geometry data, PIC (particulate inorganic carbon), POC (particulate organic carbon) and C_N (number of coccoliths per cell) thresholds for classifying the proportion of recently- and ready-to-divide cells in a population, based on the complete coccosphere geometry dataset from all experiment days. Summary statistics for both growth phases are shown in Table S1. The full dataset of experimental conditions, daily growth rates and coccosphere geometry measurements from each individual coccosphere is available as Sheward et al. (2016) at Pangaea.de, <https://doi.pangaea.de/doi:10.1594/PANGAEA.865403>.

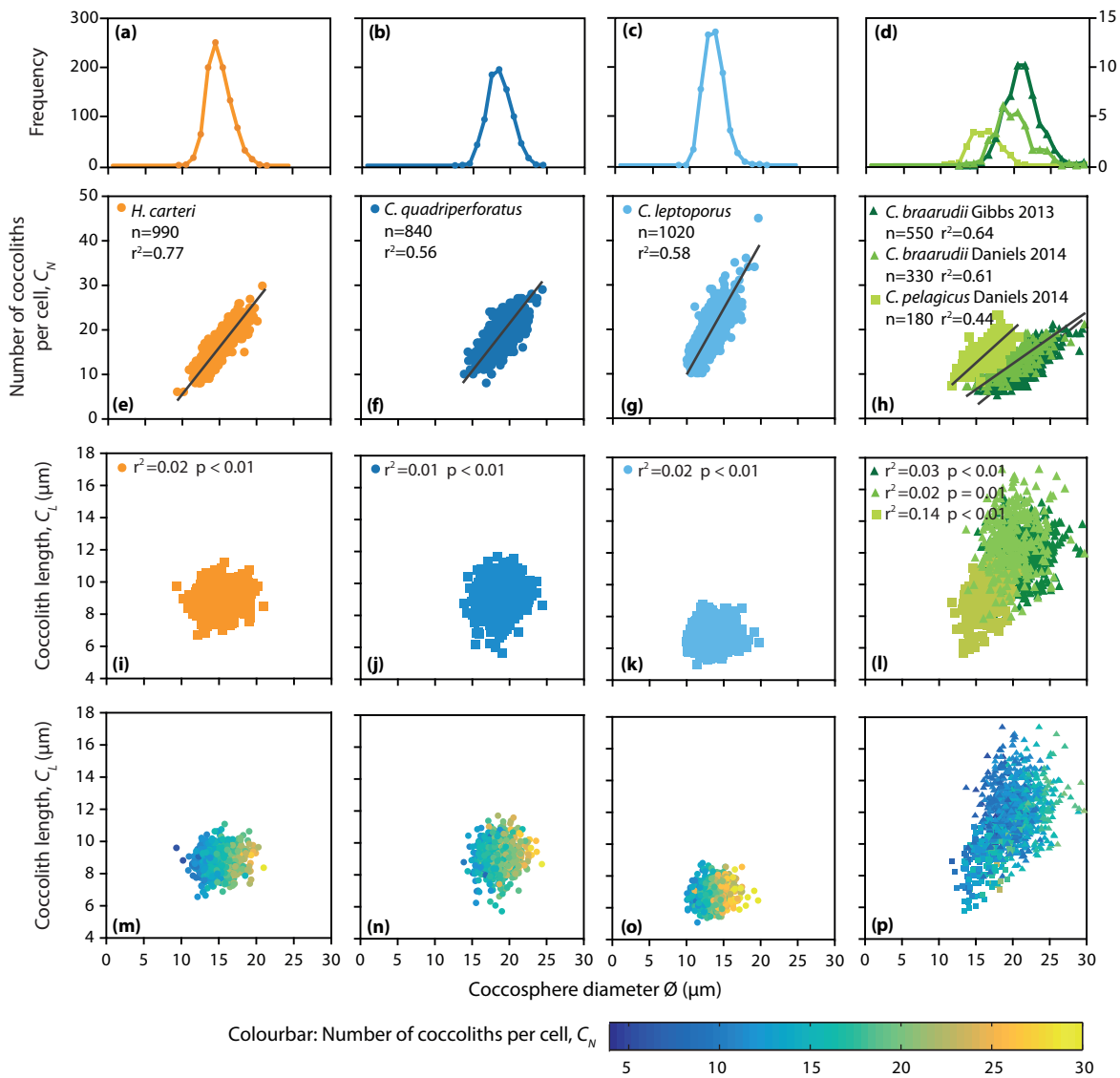
Parameter		<i>Helicosphaera carteri</i>	<i>Calcidiscus leptoporus</i>	<i>Calcidiscus quadriperforatus</i>	<i>Coccolithus pelagicus</i>	<i>Coccolithus braarudii</i>
Number of values		990	1020	840	180	880
Coccosphere diameter, \emptyset μm	Min	9.35	10.02	13.84	11.74	13.66
	Mean	15.01	13.28	18.56	16.12	20.49
	Max	20.90	19.72	24.39	20.80	29.68
Cell diameter, \emptyset μm	Min	6.53	6.39	8.64	7.94	9.92
	Mean	12.01	9.90	13.72	12.89	16.36
	Max	17.99	16.54	18.81	18.11	25.83
Coccolith length, C_L μm	Min	6.70	5.02	3.67 5.67	5.68	7.87
	Mean	8.89	6.72	8.12 9.10	8.95	12.21
	Max	11.22	8.76	11.67	11.59	17.32
Coccoliths per cell, C_N μm	Min	6	10	8	7	5
	Mean	16	19	18	14	12
	Max	30	45	29	23	24 20
PIC pmol C cell^{-1}	Min	7.79	3.27	6.09	2.16 4.15	4.15 6.97
	Mean	24.48	13.00	30.69	17.28	43.62 35.89
	Max	54.95	36.58	80.19	211.3 42.08	42.08 147.80
POC pmol C cell^{-1}	Min	1.94	1.82	4.27	6.29	3.36
	Mean	11.21	6.57	16.26	13.78	27.77
	Max	33.64	26.55	38.13	93.19	34.27
Recently divided cells $C_N \leq$		$C_N \leq 12$	$C_N \leq 14$	$C_N \leq 14$	$C_N \leq 11$	$C_N \leq 8$
Ready to divide cells $C_N \geq$		$C_N \geq 21$	$C_N \geq 23$	$C_N \geq 25$	$C_N \geq 18$	$C_N \geq 16$
Reference		This study	This study	This study	Sheward et al. (2014)	Gibbs et al. (2013); Sheward et al. (2014)

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Figures



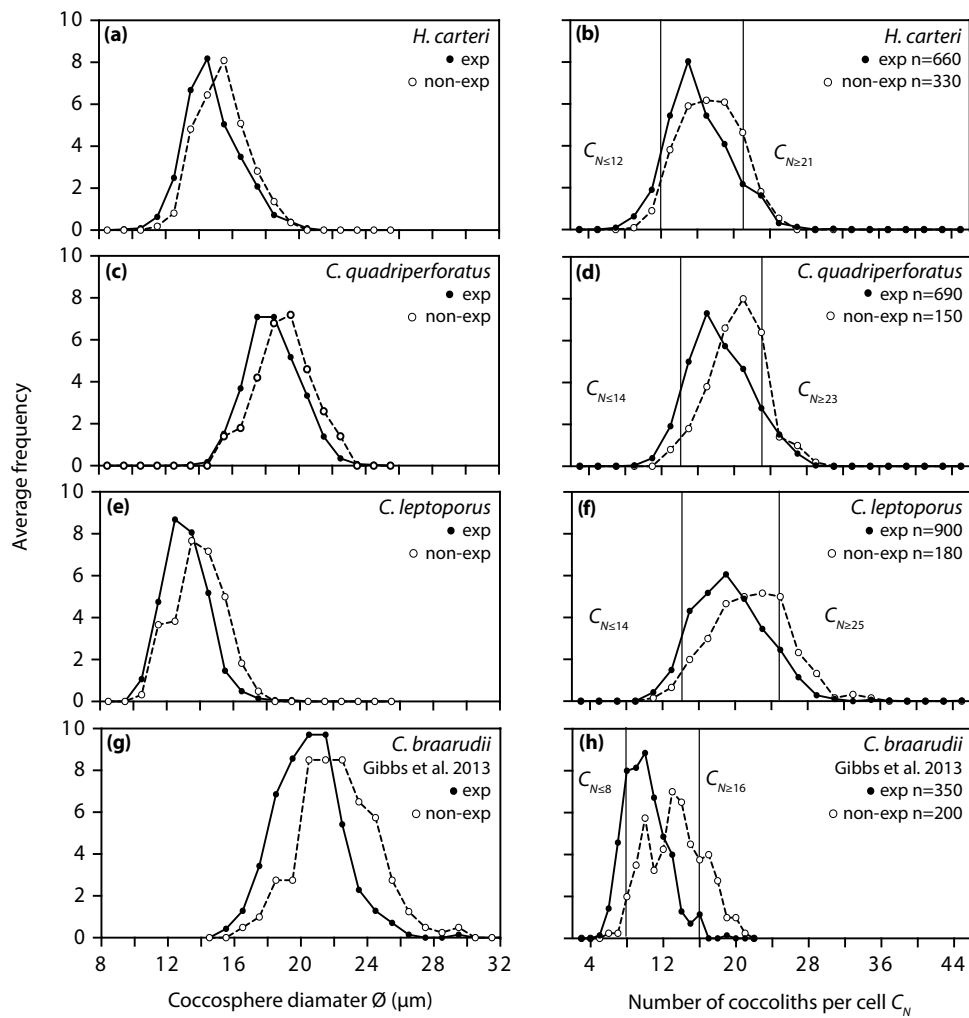
765 **Figure 1.** Light microscope image of a *C. quadriperforatus* coccosphere illustrating the coccosphere geometry terminology used in this study and the size measurements made on each individual coccosphere. After counting the number of coccoliths per cell (C_N), images are taken of (a) an in-focus, representative coccolith on either the top or bottom surface of the coccosphere from which coccolith length (C_L) is measured, and (b) a cross-sectional view from which the coccosphere diameter (\emptyset) and internal coccosphere diameter, **assumed to represent** cell diameter (\emptyset), are measured.



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Figure 2. The full range of coccosphere geometry in *H. carteri*, *C. quadriperforatus* and *C. leptoporus*. (a)–(d) Histograms of coccosphere diameter (\emptyset) calculated for frequency bins of $1\mu\text{m}$ size. Note the different frequency scale in plot (d). (e)–(h) Number of coccoliths per cell (C_N) against \emptyset showing a strong and statistically significant ($p < 0.0001$) positive relationship. The reduced major axis regression lines have slopes of *H. carteri* – 2.10 (bootstrapped 95 % confidence interval, CI [2.03, 2.17]); *C. quadriperforatus* – 2.11 (95 % CI [2.02, 2.20]); *C. leptoporus* – 3.01 (95 % CI [2.87, 3.14]); *C. braarudii* Gibbs et al., 2013 – 1.35 (95 % CI [1.27, 1.43]); *C. braarudii* Daniels et al., 2014 – 1.17 (95 % CI [1.09, 1.25]); *C. pelagicus* Daniels et al., 2014 – 1.50 (95 % CI [1.31, 1.67]). (i)–(l) Coccolith length (C_L) with \emptyset . (m)–(p) C_L and \emptyset with data points coloured by C_N . For comparison purposes, we include data for *C. braarudii* and *C. pelagicus* that can be found in Gibbs et al. (2013) and Sheward et al. (2014) accompanying Daniels et al. (2014).

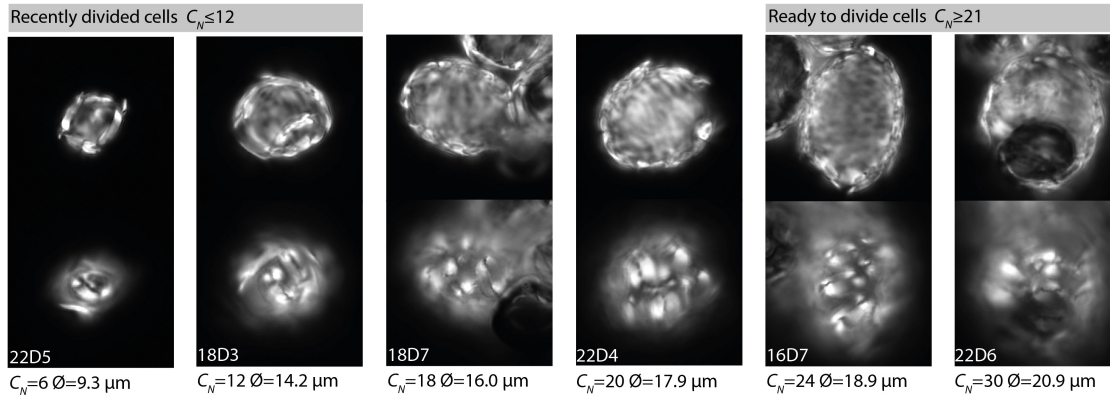
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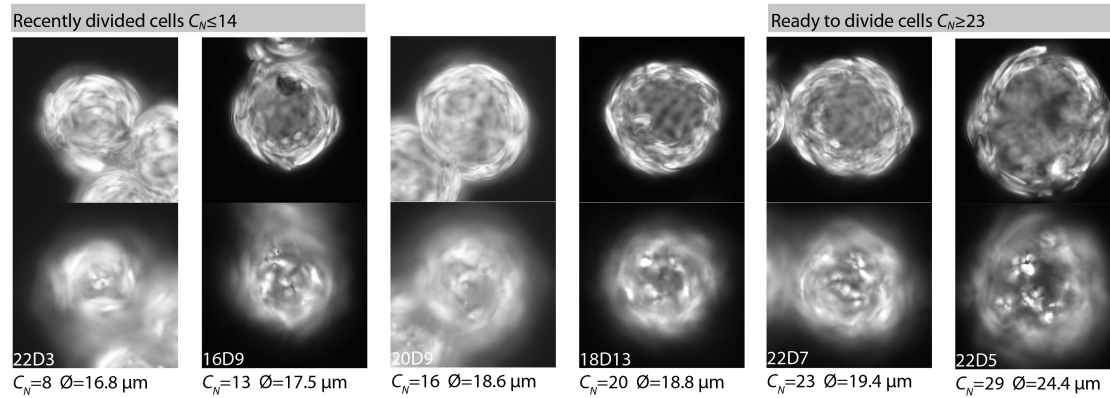
785 **Figure 3.** Frequency of coccosphere diameter (\emptyset) and number of coccoliths per cell (C_N) for experiment days in exponential growth (solid line) and experiment days no longer in exponential growth (dashed line), averaged across all temperature treatments. (a)–(f) *H. carteri*, *C. quadriperforatus*, and *C. leptopus* data from this study. (g)–(h) is a reproduction of *C. braarudii* experiment data from Gibbs et al. (2013) SI Figure 1e. and 1.f for comparison purposes. The lines drawn on C_N plots indicate cells that are recently divided and ready-to-divide/non-dividing, based on the 10th and 90th percentiles of the complete species C_N data shown in Fig. 2.

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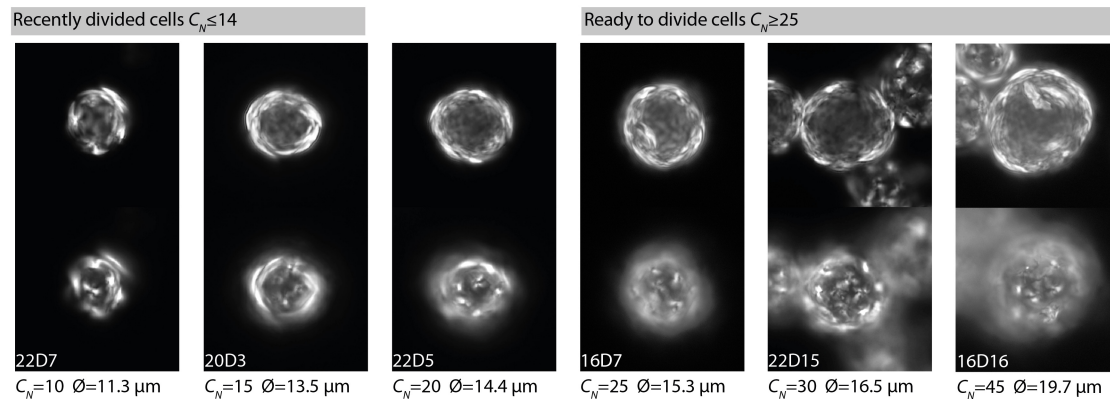
(a) *Helicosphaera carteri*



(b) *Calcidiscus quadriperforatus*



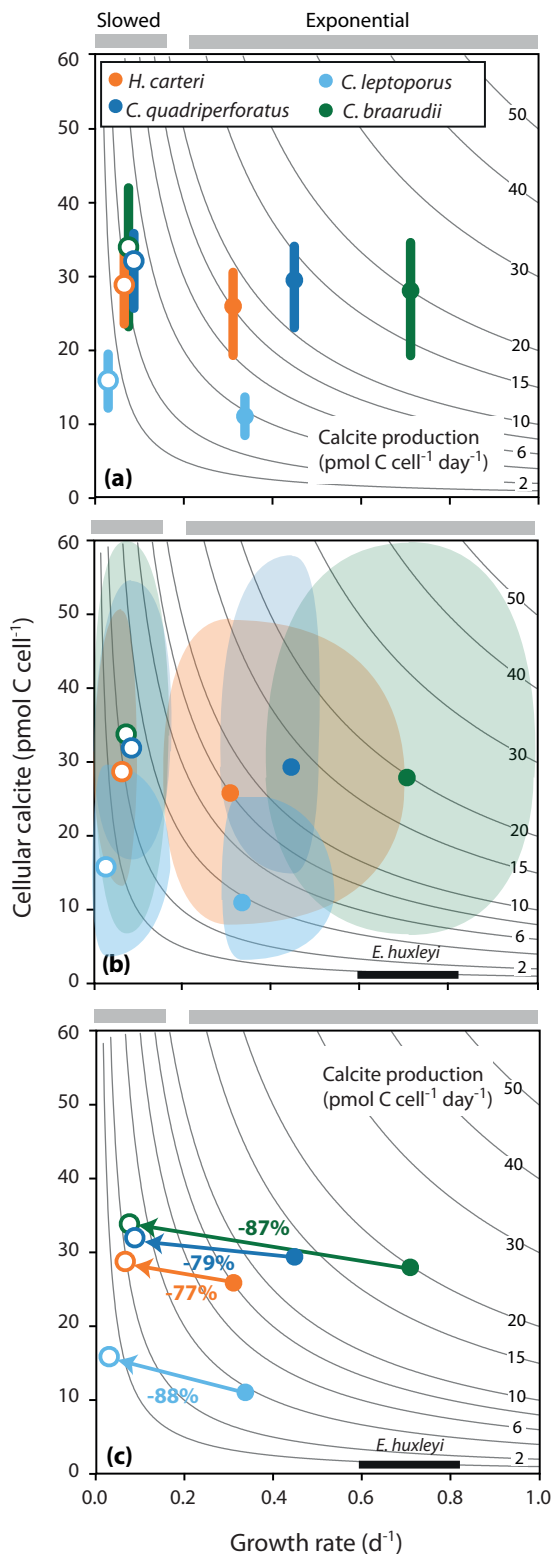
(c) *Calcidiscus leptoporus*



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Figure 4. Light microscopy images illustrating the full range of cell geometry observed across all experiment days within cultures of (a) *H. carteri*, (b) *C. quadriperforatus*, and (c) *C. leptoporus* at 16–22 °C. The upper image of each pair shows the cross-sectional view of the cell from which coccosphere diameter and cell diameter are measured. The lower image of each pair shows a coccolith-focused view of the cell from which coccolith length is measured. Number of coccoliths per cell (C_N) and coccosphere diameter (\emptyset) are given for each cell. End-member geometries illustrating recently-divided and ready-to-divide cells are shown, based on their C_N and \emptyset . Both exponential-phase and non-exponential-phase cultures will contain some recently-divided and some ready-to-divide cells, but the proportion (%) of each will differ depending on growth phase, as shown in Fig. 3 and Fig. 6. A reference code for the experiment day that the image was taken from is also given. 22D7 would be a cell from Day 7 of the 22 °C experiment as an example. All images are to the same scale.



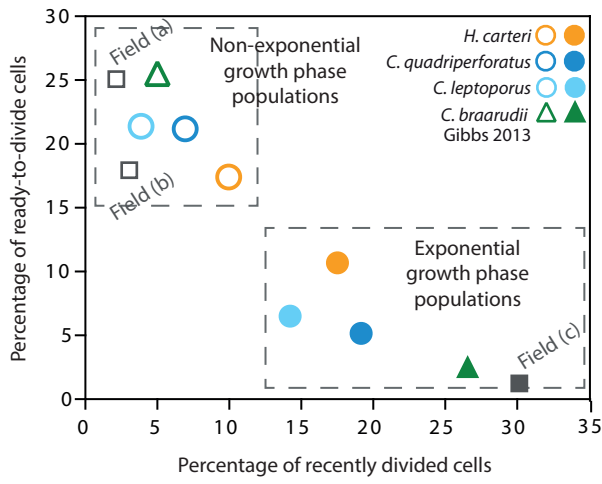
805 **Figure 5.** Calcification rates in *Coccolithus*, *Calcidiscus* and *Helicosphaera* at 22 °C. (a) mean and 25th to 75th percentile of cellular calcite
for cultures dividing exponentially (mid-exponential-phase days, see Table S1; filled circles) and cultures no longer maintaining
exponential growth (unfilled circles). (b) Range in cellular calcite, daily growth rates and calcite production observed across the
experiment. (c) Percentage decrease in mean calcite production when cultures can no longer divide exponentially. The black box in (b) and
810 (~0.2-0.8 pmol C cell⁻¹ day⁻¹) for *E. huxleyi* for comparison (Balch et al., 1996, Poulton et al.,
2010).

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835 **Figure 6.** Contrasting exponential and non-exponential phase culture populations based on the percentage of recently divided and ready-to-divide cells within the population, as characterised by C_N thresholds specific to each species (Fig. 3; Table 1). Mean percentages for exponential days are shown as filled data points and the mean non-exponential experiment day percentages are shown as unfilled data points. Also indicated (grey squares) are the characteristic percentages of three *Coccolithus* field population datasets presented in Gibbs et al. (2013) - Field (a) is Scotland, Field (b) is Iceland non-bloom (both experiencing slowed growth), and Field (c) is Iceland bloom experiencing rapid growth.