Biogeosciences Discuss., 6, C4531–C4547, 2010
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Biogeosciences Discussions

Interactive comment on "Individual and interacting effects of pCO_2 and temperature on *Emiliania huxleyi* calcification: study of the calcite production, the coccolith morphology and the coccosphere size" *by* C. De Bodt et al.

C. De Bodt et al.

cdebodt@ulb.ac.be

Received and published: 24 February 2010

We would like to thank the anonymous referees for their useful comments regarding our manuscript. Please find our reply to the comments of the first and the second referee, respectively, in the text below.

Response to comment of referee #1

There are two major comments; one concerning the carbonate system and one concerning the study design. The first one can be easily settled since we have measured

two parameters of the carbonate system (pH and TA) during the entire course of the experiment. Here, we present those parameters as well as the phosphate evolution, as an example of nutrient consumption. The evolution of parameters is shown here as information and in the future version of the manuscript we will add a table with the parameters in the beginning and at the end of the culture experiments (as proposed by the second referee). We will also justify the experimental setup chosen. Smaller and technical comments will be corrected in the future version of the manuscript and will be helpful to improve the discussion.

Specific comments:

1) One major comment of the reviewer is that we did not show parameters of the carbonate system.

The in situ pCO2 is obtained by bubbling gases with the target CO2 during the entire course of the experiment. Biological activity by equation (1) and (2) of our manuscript can modify this in situ pCO2. Then, pCO2 was calculated from pH and TA using the CO2SYS Package (Lewis and Wallace, 1998). The dissociation constants for carbonic acid given by Mehrbach et al. (1973) as refitted by Dickson and Millero (1987) were used. pH and TA were measured every day or every two days.

Parameters of the carbonate system: pCO2 was kept relatively constant during the course of the culture experiments but some variations occured during the development (growth and calcification) of E. huxleyi. It was rather difficult to maintain a constant pCO2 at the beginning of the experiment when biological activity was important, which was enhanced in the future CO2 treatments. Finally, the calculated pCO2 values in our cultures were on average always, to some extent, higher than those of the gas bottles. At 13°C, in the low CO2 cultures, pCO2 was maintained at $\sim$ 230 ppmV, in the present CO2 cultures at $\sim$ 420 ppmV and in the future CO2 cultures at $\sim$ 770 ppmV (Fig. 1). At 18°C, pCO2 was increased to $\sim$ 490 ppmV in the present CO2 cultures and to $\sim$ 840 ppmV in the future CO2 cultures (Fig. 1). pH measurements were carried

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out with a combined pH electrode (Metrohm), calibrated on the Total Hydrogen Ion Concentration Scale, using TRIS (2-amino-2-hydroxymethyl-1,3-propanediol) and AMP (2-aminopyridine) buffers prepared at a salinity of 35 following Dickson (1993), and using the pK for TRIS given by DelValls and Dickson (1998), and that for AMP given by Dickson (1993). Like the pCO2, the pH varied slightly in our cultures due to biological activities (Fig. 2a). An initial increase in pH was observed in each culture during photosynthesis, followed by a decrease in pH concomitant to biogenic calcification. The material and methods for the measurement of TA is detailed in section 2.3. of our manuscript. TA was constant at the beginning of the experiments and then decreased in all batch cultures, indicating the onset of calcification by E. huxleyi (Fig. 2b). In most of the cultures, TA reached a relatively constant value towards the end of the experiment. At 13°C, the greater drop observed was in the future CO2 treatment with 985 [978; 991] μmol kg-1. A difference between the duplicate cultures was observed in the present CO2/13°C treatment. At 18°C, initial TA was 2209 [2201; 2216] μmol kg-1 in the future CO2 treatment and decreased reaching a minimum of 1210 [1193; 1227] μmol kg-1. TA was 2209 [2202; 2216] μmol kg-1 at the beginning of the present CO2/18°C experiments and the minimum value obtained was 902 [842; 962] μmol kg-1, representing the highest consumption of TA in all experiments conducted in this study. The evolution of TA was variable between the duplicate cultures, but TA reached similar level at the end of the experiment. DIC was just measured once a week to check the pCO2 measurement given with pH and TA.

2) The second major comment of the reviewer is why the low pCO2 / 18°C condition was not included into the study design.

We did not perform such a culture experiment because we assumed that the combination of low CO2 levels and high temperature could not realistically be expected in natural circumstances in the near future. Nonetheless, it is true that for purely statistical reasons a complete set of treatments would have been more straightforward to analyse.

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3) Report degrees of freedom plus the associated test values (t-values or F-values, respectively) and use non-parametric tests.

This will be done for the future version of the manuscript.

4) Page 11138, lines 11-15: I assume that in line 14 “low” should be “future” (otherwise the same difference would be significant and non-significant). However, the significant effect of the one-way ANOVA is pretty unsurprising given the differences already evaluated with the t-tests. In fact, this analysis does not provide any news. This is an instance where the opposite way is of more value: first the ANOVA, second (if the ANOVA was significant) follow-up tests to explore the source of the ANOVA (main) effect.

Change the paragraph with: At 13°C, a one-way ANOVA indicated a significant effect of the pCO2 ($p<0.05$) with a decrease in the Δ[PIC]:Δ[cell] ratio with increasing pCO2 at 13°C. The highest cell abundance-normalized PIC ratio was found in the low CO2 treatment and was significantly different from the present CO2 treatment (by 19%) and from the future CO2 treatment (by 46%) (t-tests, $p<0.05$) (Fig. 4b). Concerning the statistical remark, we will perform a post-hoc test (Tukey or other) in the revised version of the manuscript.

5) Page 9, line4-7: I do not understand what the authors mean “by comparing the slope of the significant linear regression”.

Table 2 will be removed in the revised manuscript version since this table did add any information to what is already shown in the Figure 4 of the manuscript.

6) Growth and ‘health’ of Emiliania huxleyi: I have doubts regarding the ‘health’ status of E. huxleyi since the growth rates indicated in Table 1 (max. $\mu = 0.1$ d-1) are far to low for the experimental temperature and light conditions. At similar conditions Buitenhuis et al. (2008) report growth rates of approx. 0.8 d-1 at 13°C and 1.2 d-1 at 18°C under nutrient replete conditions. “Aged surface post-bloom seawater” without addition

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of micronutrients was used as growth media, could this have lead to a limitation be one or several micronutrients? Possibilities leading to this low growth rate should be discussed as well as the effects of phophate limitation during the stationary growth phase.

It is true that our growth rates were slower than those previously reported for E. huxleyi (Trimborn et al., 2007; Buitenhuis et al., 2008; Barcelos e Ramos et al., 2009). These culture experiments used culture media enriched with trace metals and vitamins such as f/2 medium (Guillard, 1975; Guillard and Ryther, 1962) or K/5 medium (Keller et al., 1985). It is thus possible, as pointed out by the reviewer, that the omission of trace element (iron, zinc or cobalt) addition or also the lack of vitamins contributed to the slower growth of our cultures. This is supported by measurements made on cultures (of the same E. huxleyi strain) that were conducted in our laboratory with and without the addition of trace metals and vitamins (Carbonnel, V., pers. com.). In the future version, we will add a short literature review about typical growth rates reported for culture experiments and add a discussion about the possible cause of our low growth rates. Since the same nutrients enrichment was applied in our culture experiments, the slower growth rate would not affect the main conclusions of our experiments. Growth rates were measured during the exponential growth when macronutrients were not limiting. Macronutrient limitation (during the stationary growth phase) has an effect on the biomass production and on the physiology of the cells and a short discussion can be added on this subject in the future version of the manuscript.

7) Definition of the 'exponential growth phase' and the 'calcification phase': How was the exponential growth phase defined and on what basis where the data points pooled to calculate the regressions of POC and PIC per cell in Table 2? There are contrary statements since on page 11135, line 20, it is written that: "The duration of the exponential growth phase varied between 7 (low CO2/13°C treatment) and 15 d (present and future CO2/18°C treatment) from one culture experiment to another." but Table 2 indicates a exponential growth phase for the low CO2/13°C treatment of 26 days (d8

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to d34); there are similar contraries for the other treatments. + The 'calcification phase' was defined when the calcite saturation state (Omega) was above one (Table 2 caption). Does that mean that on the other days (which are not included in the calculation of PIC per cell) Omega was below one? It is true that there are contrary statements concerning the exponential growth phase. On page 11135, line 20, the exponential growth phase refers to the phase when Chl a concentration increases exponentially under nutrient replete condition.

The error is in Table 2 of the manuscript (which will be removed in the revised version of the manuscript); the data points used to calculate the regressions of POC per cell are not taken during the exponential growth phase but during the organic carbon producing phase (from the beginning of the increase in POC concentration until POC concentration or cell density decrease). The Δ[PIC]: Δ[cell] ratio was determined during the calcification phase which refers to the phase during which inorganic carbon is produced and before omega calcite falls below 1. Calcification led to a significant decrease in Ωcal during each culture experiment due to the consumption of CO32-. Ωcal reached values $\leq$ 1 at the end of the culture experiments.

8) Nutrients and total alkalinity: Nutrient consumption was used for alkalinity correction. How were the nutrients measured and can you provide data on the nitrate and phosphate consumption under the different CO2 levels and temperatures.

Samples for nutrient measurements were filtered through Nuclepore filters (0.4 μm pore size) and filtrates were stored at -20°C until analyses. NO3 was determined colorimetrically with a Technicon Autoanalyzer system and PO4 was measured manually with a spectrophotometer, both analyses following Grasshoff et al. (1983). The growth of E. huxleyi was accompanied by a consumption of nutrients. The drawdown of PO4 is almost the same between CO2/temperature treatments (Fig. 3a). Just at 18°C, the drawdown was much faster in one of the duplicate at future CO2. An important decrease in PO4 was observed the first 10 days, after which the PO4 levels were close to 0 from day 20 (d20) onwards.

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9) Figure 7 and calcification rates: The reported calcification rates of <1.1 pgPIC per cell and day (derived from Fig. 6?) are far lower than commonly reported in the literature (10pgPIC per cell and day). Unfortunately, the authors provide no discussion point on that, rather for the concomintant low POC production rates (since a PIC:POC ratio of 2 is reported).

Growth rate influence the PIC production (or cellular calcification rate) as well as the POC production since they were obtained by multiplying growth rates with cellular carbon content. That will be mentionned in the next version of our manuscrit and our production rates will be compared with previous observations. Please plot the error bars (1SD) for the individual data points in Fig. 7. This will be done for the future version of the manuscript.

10) Page 11140, line 23: "The smaller size of coccosphere at 18°C (Fig. 6) is likely to be at the origin of the lower chl-a per cell ratio at higher temperature." Why should lower chla content per cell lead to a smaller size of the coccosphere. What about the organic and inorganic carbon content of the cell?

What was meant is that we observed lower chl-a content per cell at 18°C which could be attributable to the smaller cell size observed at 18°C. The organic and inorganic carbon content of the cells was also lower at 18°C. The relation between cell size and calcification is discussed in the paper.

11) Page 11141, line 19: Please clarify this paragraph. Anyway, what is meant be the "extracellular release of primary production"?

We can rework this section and better explain the extracellular release of dissolved organic matter. "The DIC then consumed by the algal cell cannot be further metabolised into cell constituents, such as proteins or nucleic acids, because of the limiting nutrients (N and P) and is therefore released as dissolved carbon-rich organic material, such as polysaccharides."

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Technical comments:

Report the salinity: It is 35.6

Be consistent in reporting the unit of POC and PIC, it switches between ‘gram’ and ‘mol’. This will be done for the revised version of the manuscript.

Fig. 2: Clarify the legend, diamonds are missing. This will be done for the revised version of the manuscript.

Fig. 2 caption: “Squares and diamonds represent the duplicate culture experiment”. There are no squares in this figure. This will be adapted in the revised version of the manuscript.

Page 11139, line 9: “No interactive effect of pCO2 and the temperature was...” Change ‘interactive’ to ‘interaction’. This will be done for the revised version of the manuscript.

Fig. 6 caption: What is meant by the maximum and minimum mean values (how are they calculated)? We will adapt the figure caption to clarify possible ambiguities as follows (bold text): Figure 6. Mean particle size of CSP for each treatment. The central marker denotes the mean, the standard error is given by the box boundaries, and the whiskers represent the minimum and maximum mean values of the size measurement on three time points in duplicate cultures (n=6). The white box represents the low CO2 treatment, the grey shaded boxes the present CO2 treatments, and the black boxes the future CO2 treatments. The right-most two boxes represent the 18 °C treatments.

Response to comment of the referee #2

1) The major concerns of the second referee are that it is unclear from which days the results used to compile each of the figures originate, and that samples taken at different times from the batch culture and particularly throughout the stationary phase cannot be compared as “replicates” in any realistic way. The methods section states, that samples were taken every 2-3 days for analysis of PIC and POC, chl-a, and cell density etc. Yet, in the results section as for the figure captions it is not clear which data

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from which sample are used in the figures. It is well documented that the PIC/POC ratio of cells can evolve throughout a batch culture, and so therefore it is essential that like be compared with like i.e. samples at e.g. 30% and 60% of the maximum population should be compared, or even samples with similar growth rates. It is essential that this sort of data be presented and discussed before this manuscript can be finally published.

For figures 5 (coccolith morphology) and 6 (cell size) the sampling time is precised in the materials and methods. For the PIC and POC ratio, the delta t was presented in table 2 (which will be removed in the future version). For Figures 4 and 7 the sampling time will be defined in the next version of the manuscript. We will calculate the PIC:cell and POC:cell ratio as well as the PIC:POC ratio during the exponential growth phase (between 50 and 100% of maximum cell abundance)

2) I also found a lot of the discussion of the PIC and POC concentrations and chl-a concentrations very unhelpful. The actual data of interest should be presented as cell normalised in terms of C fixation rates per cell per day (either for PIC or POC). There is quite a lot of unnecessary discussion about increasing POC, PIC and chl-a concentrations through an exponentially growing culture!

The pmol PIC cell-1 d-1 is presented in figure 7 and in the revised version of the manuscript we will also present POC in pmol cell-1 d-1. We can also remove the graph in Figure 2 and present these results in a table such as for the parameters of the carbonate system.

3) I think it would be very helpful to include a table which details the carbonate chemistry parameters both at the beginning and the end of the experiments (i.e. DIC, TA, pH, pCO2, CO32-, HCO3- and saturation state) just so that the reader is able to see what changes in saturation state occur with temperature etc and one can independently see how these parameters change throughout the experiment.

Instead of presenting the evolution of the parameters of the carbonate system during

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our culture experiments as we show for example in figure 2, we will add a table as the second referee proposed.

4) I am interested in what was done regarding agitation of the cultures. Mixing of the cultures is essential both during the experiment and at a minimum before sampling, but there are little details regarding what sort of shaking or disturbances were applied.

The sampling was done every morning at the same time and bottles of cultures were gently shaken manually before sampling.

5) On Figure 2, I think that cell normalised chl-a should be added as a series of panels.

The cell normalised chl-a is presented in Figure 4. In the future version of the manuscript, we will calculate this ratio during the exponential growth phase as for the PIC:cell and POC:cell ratios (between 50 and 100% of maximum cell abundance). We will determine how the Chl-a concentration per cell differs between the CO2/temperature treatments by statistical analysis.

6) Section 3.4 The comparison of coccolith morphology is interesting. I wonder if there is a compilation of the standard "expected' rates of malformation in a laboratory culture. Certainly these liths can be produced in the field and seem to be a standard feature of coccolithophores not always getting it right. I also wonder about the degree of subjectivity in the 4 categories of malformation which are presented.

We are not aware of a compilation of expected rates of malformation of cell surface-attached coccoliths in laboratory culture or natural circumstances, but this could be a start (but see Langer et al., 2006). Although malformed (detached) liths occur in the field, we compared the relative occurrence of degrees of malformation of attached coccoliths between different experimental treatments without using a natural baseline, deemed unnecessary in this experimental context (yet interesting indeed). As the categories used to classify the degree of coccolith malformation were defined a priori (see 2.4.) instead of using reference images (Langer et al., 2006), we reduced the amount

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of possible subjectivity to the very minimum.

7) Section 4.3: The authors have some apparently robust differences between the size of the cells in each of the treatments particularly with respect to temperature. However, it appears they have not really explored their data to the full here. Do the values from the Coulter Counter sizer agree with what can be measured on SEM. Did they attempt to add a few drops of acid to the cultures being counted and sized which can eliminate the liths and yield and lith-free cell size? This would allow us to know really whether the changes in cell size reported are due to changing lith numbers on the cells, or due to a fundamental change in the organic cell size.

First, we did not perform coccosphere size measurements on SEM images because it is quite inaccurate to perform such radius measurements on cells unevenly covered by liths compared to the impedance-based size measurements of thousands of particles obtained by the Coulter Multisizer. Secondly, we did not attempt to dissolve the attached liths on the coccosphere to measure the naked cell's radius. Although this procedure seems quite interesting and even could be added as a suggestion for further research in the discussion, it would need additional testing since the effects of an acid treatment on naked cells are unknown.

8) Section 4.4 The authors still talk about the apparently contradictory results from Riebesell et al., 2000 versus Iglesias-Rodriguez, 2008. There is an additional paper from Langer et al., 2009 which addresses strain-specific affects of different E. huxleyi strains under different CO2 conditions and it appears that strain selection in culture may go some way to reconciling some of these differences. I would suggest that a reference to this paper is useful here.

Indeed, Langer et al. (2009) explained the different responses observed by the fact that different strains were used during the various studies. These authors showed that 4 strains of E. huxleyi responded differently to different CO2 levels and proposed that the strain specific response have genetic bases. We will add this reference to the revised

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version of the paper.

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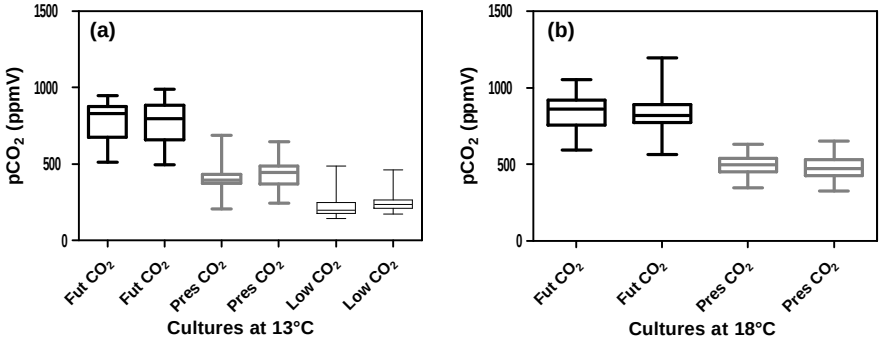

Figure 1. Mean calculated pCO_2 values during the duration of each culture at 13°C and (b) at 18°C. The central marker denotes the mean, the standard error is given by the box boundaries, and the whiskers represent the minimum and maximum mean values. The thick black boxes represent the future CO_2 treatments, the grey boxes the present CO_2 treatments and the thin black boxes the low CO_2 treatment.

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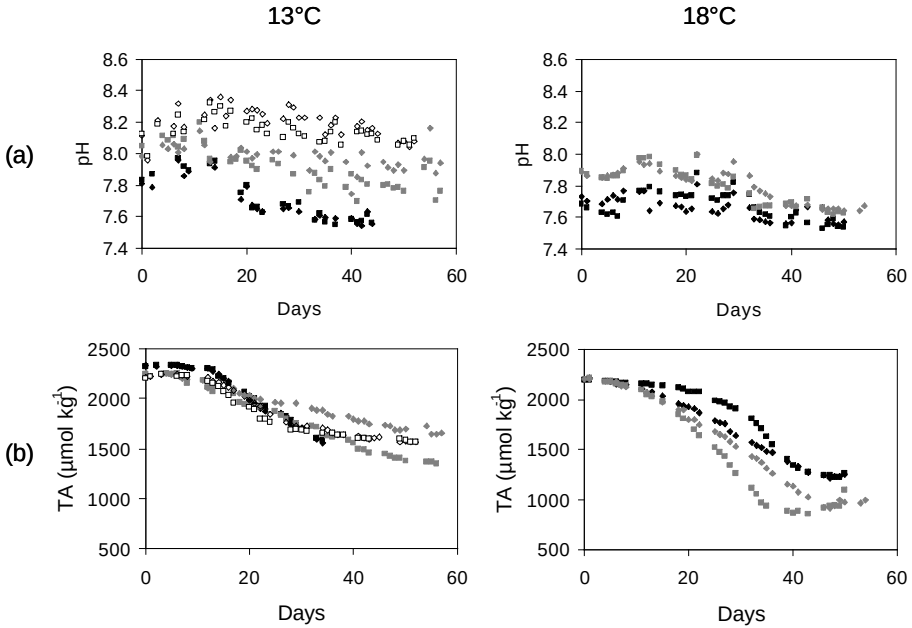

Figure 2. Evolution with time of (a) pH and (b) TA during the batch culture experiments. Open symbols represent low CO_2 treatment, grey symbols present CO_2 treatment and black symbols future CO_2 treatment. Squares and diamonds correspond to the duplicate culture experiments. The left panels presents the culture experiments at 13°C and the right ones those at 18°C.

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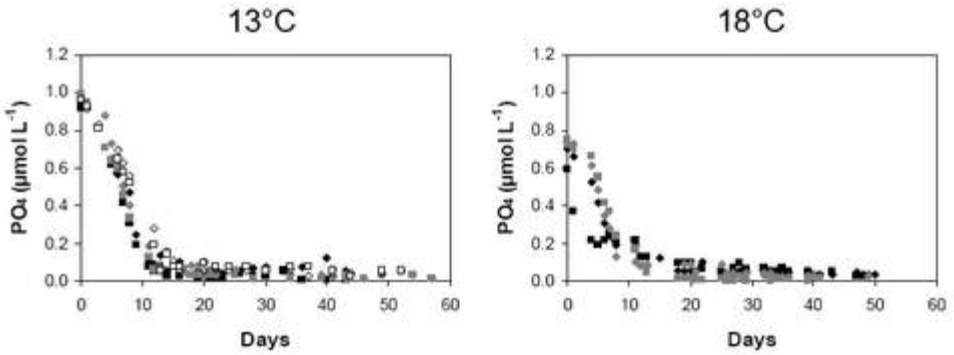

Figure 3. Evolution with time of phosphate (PO_4) concentrations during the batch culture experiments. Open symbols represent low CO_2 treatment, grey symbols present CO_2 treatment and black symbols future CO_2 treatment. Squares and diamonds designate the duplicate culture experiments. The left panel presents the culture experiments at 13°C and the right one those at 18°C.

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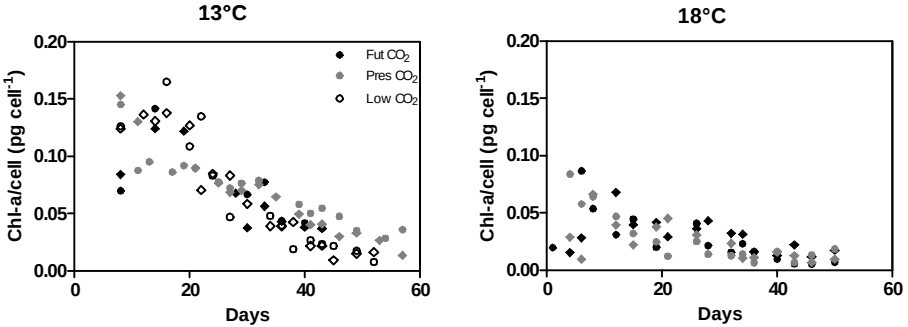

Figure 4. Evolution with time of Chl-a per cell during the batch culture experiments. Open symbols represent low CO_2 treatment, grey symbols present CO_2 treatment and black symbols future CO_2 treatment. Squares and diamonds designate the duplicate culture experiments. The left panel presents the culture experiments at 13°C and the right one those at 18°C.

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