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CO_2 mediation of adverse effects of seawater acidification in *Calcidiscus leptoporus*

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[1] The coccolithophore *Calcidiscus leptoporus* (strain RCC1135) was grown in dilute batch culture at CO_2 levels ranging from ~200 to ~1600 μatm. Increasing CO_2 concentration led to an increased percentage of malformed coccoliths and eventually (at ~1500 μatm CO_2) to aggregation of cells. Carbonate chemistry of natural seawater was manipulated in three ways: first, addition of acid; second, addition of a HCO_3^-/CO_3^{2-} solution; and third, addition of both acid and HCO_3^-/CO_3^{2-} solution. The data set allowed the disentangling of putative effects of the different parameters of the carbonate system. It is concluded that CO_2 is the parameter of the carbonate system which causes both aberrant coccolithogenesis and aggregation of cells.

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1. Introduction

[2] The decrease in surface ocean pH due to anthropogenic CO_2 emissions, commonly referred to as ocean acidification [*Royal Society*, 2005], has motivated a considerable number of CO_2 perturbation experiments on marine calcifying organisms [*Fabry*, 2008]. More often than not it was found that the capacity of calcifiers to build shells is impaired by acidification of seawater. Coccolithophores, biogeochemically important, unicellular haptophyte algae, which surround themselves with elaborately crafted calcite platelets (coccoliths), are no exception to this rule. It was demonstrated that coccolith morphogenesis of, e.g., *Calcidiscus leptoporus* is hampered at high CO_2 concentrations and low pH [*Langer et al.*, 2006]. Although methods to manipulate the carbonate system of seawater differ between different studies, all experiments conducted so far, with the exception of a study by *Buitenhuis et al.* [1999], see Discussion for further details, have one feature in common: several parameters of the carbonate system were changed in concert. In particular, the increase in CO_2 concentration always entailed a decrease in pH, and in carbonate ion concentration, and so a decrease in supersatu-

Table 1. Data Set Derived From the Experiments[a]

	Experiment					
	1	2	3	4	5	6
Carbonate Chemistry						
pCO_2 (μatm)	215	917	969	1269	1634	1532
CO_2 (μmol kg^{-1})	7.1	30.1	31.8	41.6	53.6	50.3
HCO_3^- (μmol kg^{-1})	1879	8239	2090	2084	2057	4571
CO_3^{2-} (μmol kg^{-1})	336.3	1515	93.4	70.2	53	275
DIC (μmol kg^{-1})	2222	9783	2216	2196	2163	4897
TA (μmol kg^{-1})	2720	11439	2354	2291	2222	5178
pH, total	8.33	8.34	7.72	7.6	7.49	7.86
Omega ($\Omega_{calcite}$)	8.22	37	2.28	1.72	1.29	6.72
Rates and Quotas						
Pg PIC cell^{-1}	91.1	127.8	111	100	181.9	87.6
SD	5.9	12.02	4.1	18.26	20.3	16.1
Pg POC cell^{-1}	59.8	71	80	65.3	171.8	136.5
SD	7.1	12.05	3.3	12.52	16.0	23.1
Pg PIC cell^{-1} d^{-1}	63.8	75.8	75.9	63.4	62	24.8
SD	4.1	7.13	2.8	11.8	6.9	4.6
Pg POC cell^{-1} d^{-1}	38.1	42.1	54.7	42.2	58.6	38.6
SD	5.0	7.15	2.3	8.1	5.4	6.5
PIC:POC	1.7	1.83	1.39	1.6	1.06	0.65
SD	0.2	0.30	0.1	0.66	0.1	0.1
Growth rate μ	0.7	0.59	0.68	0.65	0.34	0.21
SD	0.03	0.05	0.06	0.04	0.07	0.01
Morphology						
Normal (%)	88.6	7.6	30.6	14.3	1.4	1.1
SD	0.9	0.3	1.6	1.6	0.5	0.3
Malformed (%)	6.9	15.4	20.9	17.0	2.8	3.0
SD	1.0	2.6	2.2	2.2	1.2	0.9
Incomplete (%)	1.3	2.8	1.2	1.3	1.1	2.6
SD	0.4	0.8	1.9	0.7	0.1	1.0
Malformed and incomplete (%)	3.2	74.2	47.2	67.5	94.8	93.3
SD	0.8	3.0	2.5	1.9	1.7	1.5

[a]Values represent mean and standard deviation (SD) of triplicate culture experiments.

ration of seawater with respect to calcite (omega calcite).

[3] Therefore, it has been impossible to determine which parameter of the carbonate system causes the adverse effects. This knowledge, however, is required for a process-based understanding of acidification effects. The development of such a process-based understanding, in turn, is urgently needed in order to reliably predict the fate of calcifiers in an acidifying ocean [*Rost et al.*, 2008]. It was hypothesized that the increase in the percentage of malformed coccoliths with increasing CO_2 concentration in *Calcidiscus leptoporus*, one of the most productive coccolithophores in terms of calcite export to the sediments [*Baumann et al.*, 2004], is pH mediated [*Langer et al.*, 2006]. It is the objective of this study to test this hypothesis by means of a series of CO_2 perturbation experiments which allows identification of the parameter of the carbonate system causing hampered coccolith morphogenesis.

2. Material and Methods

[4] Clonal cultures of *C. leptoporus* (strain RCC1135, formerly known as AC365 and NS6–1, obtained from the Roscoff Culture Collection (http://www.sb-roscoff.fr/Phyto/RCC) were grown in aged, sterile-filtered (0.2 μm pore size cellulose acetate filters) North Sea seawater enriched with 880 μmol L^{-1} nitrate, 35 μmol L^{-1} phosphate, and trace metals and vitamins as in f/2 medium [*Guillard and Ryther*, 1962]. Cultures were grown under a 16/8 h light/dark cycle. Experiments were carried out at a light intensity of 400 μmol photons m^{-2} s^{-1} and a temperature of 20°C [*Langer et al.*, 2006] in an adjustable incubator (Rubarth Apparate GmbH, Germany). Salinity, measured with a conductivity meter (WTW Multi 340i) combined with a TetraCon 325 sensor, was 32. Cells were acclimated to experimental conditions for approximately 5 generations and subsequently grown in dilute batch cultures [*Langer et al.*, 2006]. Each data point presented in Table 1 and Figures 1–5 is the mean value of triplicate culture experiments.

[5] Low cell densities (<3000 cells ml^{-1}) even at the termination of the experiments resulted in the consumption of less than 5% dissolved inorganic carbon (DIC), ensuring a quasi-constant carbonate system over the course of the experiment [see also *Langer et al.*, 2006]. CO_2 levels were adjusted in three different ways. First, calculated amounts of HCl or NaOH were added to the medium, thereby changing inter alia pH. Second, calculated amounts of a HCO_3^- and a CO_3^{2-} solution were added to the medium, thereby keeping pH constant. Third, calculated amounts of HCl and HCO_3^- and CO_3^{2-} solutions were added, thereby changing pH and DIC. The volumes (per bottle used for the experiments) of $NaHCO_3$ (500 mM), Na_2CO_3 (50 mM), and HCl (1 M) solutions which need to be added to the seawater of experiment 1 in order to obtain the carbonate chemistry characterizing the seawater of the other experiments are listed in the following. Experiment 2: 30.2 ml $NaHCO_3$, 55.2 ml Na_2CO_3, experiment 3: 866 μl HCl, experiment 4: 1015 μl HCl, experiment 5: 1178 μl HCl, experiment 6: 12.7 ml $NaHCO_3$, 513 μl HCl. In general, bicarbonate and carbonate solutions were prepared using pro analysis salts provided by Merck (Darmstadt, Germany) dissolved in reverse osmosis water (conductivity 0.065 μS/cm). In particular, a 500 mM solution of $NaHCO_3$ and a

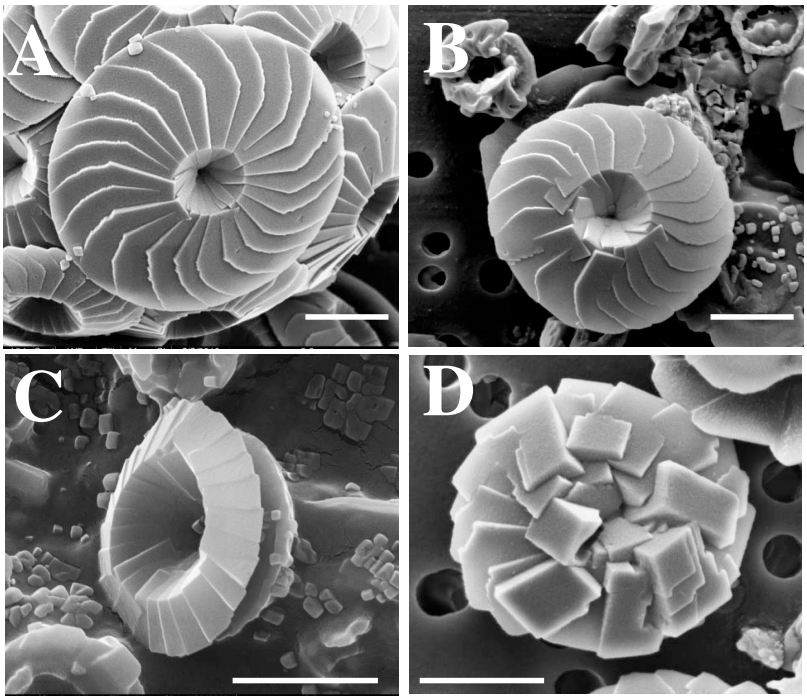

Figure 1. Scanning electron micrographs of *Calcidiscus leptoporus* coccoliths. (a) Normal. (b) Malformed. (c) Incomplete. (d) Malformed and incomplete. All coccoliths in distal view. All scale bars 2 μm.

50 mM solution of Na_2CO_3 were used. The pH of the latter was 11.21 (total scale, 20°C), while that of the former was 8.21 (total scale, 20°C). In order to prevent gas exchange with the atmosphere 2.4 l borosilicate flasks were filled without headspace and closed with Teflon lined screw caps. Regular sampling for cell counts caused only a negligible shift in the carbonate system [see *Langer et al.*, 2006].

[6] Samples for alkalinity measurements were filtered through 0.6 μm nominal pore size glass fiber filters (Whatman GF/F), poisoned with 1 ml 35 g L^{-1} $HgCl_2$, and stored in acid-washed 300 ml borosilicate flasks at 0°C. DIC samples were sterile filtered through 0.2 μm pore size cellulose acetate syringe filters and stored in acid-washed 13 ml borosilicate flasks free of air bubbles at 0°C. Samples were measured within 2 days after sampling which ensures constant DIC during storage Total alkalinity (TA) was calculated from linear Gran plots [*Gran*, 1952] after potentiometric titration (in duplicate) [*Bradshaw et al.*, 1981; *Brewer et al.*, 1986]. DIC was measured photometrically [*Stoll et al.*, 2001] in triplicate by means of a QuaAAtro autoanalyzer (Seal Analytical, Mequon, USA). Certified Reference Materials (Batch No. 54) supplied by A. Dickson were used to correct for inaccuracies of the measurements. This method is based on the acidification of the sample with sulphuric acid (H_2SO_4) to a pH < 1, which converts all carbon species to CO_2. The carrier stream is then led over a semipermeable silicone membrane where the alkaline detector stream (containing NaOH and phenolphthalein indicator) quantitatively absorbs the CO_2 of the sample. This leads to the discoloration of the indicator, which can be measured in a flow cell as

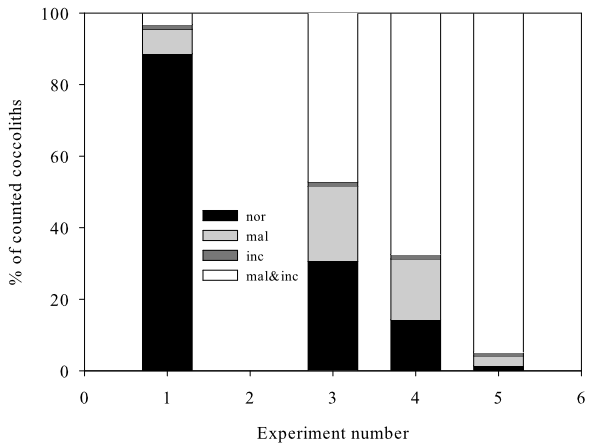

Figure 2. Percentages of normal (nor), malformed (mal), incomplete (inc), and malformed and incomplete (mal&inc) coccoliths versus experiment number. The experiments displayed represent acid/base manipulation of the carbonate system. Increasing experiment number corresponds to higher CO_2 and lower pH.

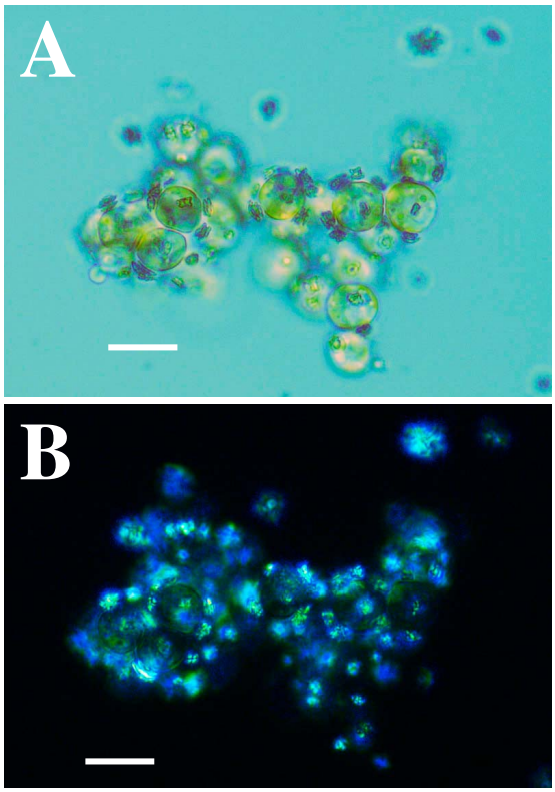

Figure 3. Light microscopy images of *Calcidiscus leptoporus* aggregates as seen in (a) bright field and (b) cross-polarized mode. The calcareous coccoliths appear bright in the cross-polarized image (Figure 3b). All scale bars 10 μm.

the change in extinction at a wavelength of 550 nm [*Stoll et al.*, 2001]. Shifts in DIC concentrations due to CO_2 exchange were prevented by opening the storage vials less than one minute prior to each measurement. Samples for pH measurements were sterile filtered through 0.2 μm pore size cellulose acetate syringe filters and measured potentiometrically using a glass electrode (Schott Instruments, Mainz, Germany) and a WTW pH meter. Calibration was performed using NBS buffers. The measured pH_{NBS} values were converted to the total scale using respective Certified Reference Materials (Tris-based pH reference material, Batch No. 2, Scripps Institution of Oceanography, USA [see also *Dickson*, 2010]). All pH values are reported on the total scale.

[7] The carbonate system was calculated from temperature, salinity, TA, pH (total scale) and phosphate using the DOS program CO_2sys [*Lewis and Wallace*, 1998]. The equilibrium constants of *Mehrbach et al.* [1973] refitted by *Dickson and Millero* [1987] were used. Samples for determination of total particulate carbon (TPC) and particulate organic carbon (POC) were filtered onto precombusted (12 h, 500°C) 0.6 μm nominal pore size glass fiber filters (Whatman GF/F) and stored at −20°C. Prior to analysis, 230 μL of an HCl solution (5 mol L^{-1}) was added on top of the POC filters in order to remove all inorganic carbon. TPC and POC

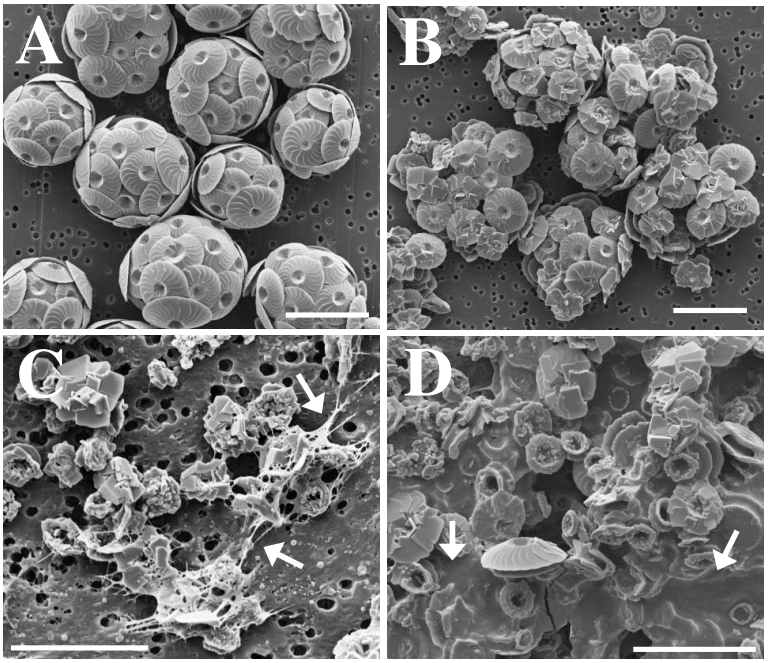

Figure 4. Scanning electron micrographic overviews of samples from (a) experiment 1, (b) experiment 4, and (c and d) experiment 6. Arrows indicate organic material of slimy appearance. All scale bars 10 μm.

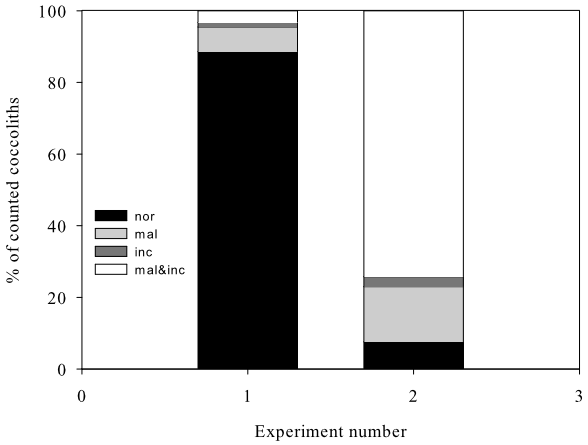

Figure 5. Percentages of normal (nor), malformed (mal), incomplete (inc), and malformed and incomplete (mal&inc) coccoliths versus experiment number. The experiments displayed represent experiments with the same pH. Increasing experiment number corresponds to higher CO_2.

were subsequently measured on a Euro EA Analyzer (Euro Vector). Particulate inorganic carbon (PIC) was calculated as the difference between TPC and POC. This method of determining PIC quota is a well established one, data quality being sufficient for the purpose of carbonate chemistry manipulation experiments [see, e.g., *Riebesell et al.*, 2000; *Zondervan et al.*, 2001, 2002; *Langer et al.*, 2006, 2007a, 2007b; *Trimborn et al.*, 2007; *Iglesias-Rodriguez et al.*, 2008; *Langer and Benner*, 2009; *Langer et al.*, 2009]. For determination of cell density, samples were taken daily or every other day and counted immediately after sampling using a Sedgwick Rafter Counting Cell. Cell densities were plotted versus time and growth rate (μ) was calculated from exponential regression.

[8] Particulate inorganic carbon production, i.e., calcification rate (P_{PIC}, pg PIC $cell^{-1}$ d^{-1}), was calculated according to:

$$P_{PIC} = \mu * (\text{cellular inorganic carbon content}) \quad (1)$$

with cellular inorganic carbon content = pg PIC per cell.

[9] Particulate organic carbon production (P_{POC}, pg POC $cell^{-1}$ d^{-1}) was calculated according to:

$$P_{POC} = \mu * (\text{cellular organic carbon content}) \quad (2)$$

with cellular organic carbon content = pg POC per cell.

[10] Samples for scanning electron microscope analysis were filtered onto polycarbonate filters (0.8 μm pore size), dried in a drying cabinet at 60°C for 24 h, then sputter coated with gold palladium. Imaging was performed with a Philips XL-30 digital scanning field emission electron microscope. Four categories were used to describe the morphology of *C. leptoporus*: "normal," "malformed," "incomplete," and "incomplete and malformed" coccoliths (for reference images for the categories, see Figure 1). An average of approximately 700 coccoliths was analyzed per sample.

3. Results and Discussion

[11] In this study three different methods of manipulating the carbonate system of seawater were employed: (1) addition of acid/base, thereby changing CO_2 concentration and pH in concert (experiments 1, 3, 4, and 5 in Table 1); (2) addition of HCO_3^-/CO_3^{2-}, thereby increasing CO_2 and keeping pH constant (experiment 2 in Table 1); and (3) addition of acid and HCO_3^-/CO_3^{2-}, thereby changing CO_2 concentration and pH in concert (experiment 6 in Table 1). In contrast to manipulation 1, however, a given change in CO_2 was accompanied by a relatively small change in pH (Table 1). A remotely similar study was carried out by *Buitenhuis et al.* [1999]. The latter study is similar to ours only insofar as *Buitenhuis et al.* [1999] also manipulated the carbonate system in different ways to decouple, e.g., CO_2 and pH changes. Everything else is fundamentally different. First, a different species, namely *E. huxleyi*, was used. Second, coccolith morphology, the central parameter in our study, was not analyzed. Third, the observed effects on, e.g., PIC production, if present, were detectable in a completely different range of carbonate chemistries, i.e., at DIC concentrations well below 1000 μM. These differences between the study of *Buitenhuis et al.* [1999] and our study render it impossible to compare results or conclusions.

[12] The addition of acid resulted in an increase in the percentage of malformed coccoliths (Figure 2 and Table 1). This is in accordance with a previous study [*Langer et al.*, 2006]. The response patterns of auxiliary parameters as determined in this study, e.g., PIC production (experiments 1, 3, and 4 in Table 1), also compare well with data given by *Langer et al.* [2006]. The highest CO_2 concentration due to acid addition in this study (experiment 5 in Table 1) led to aggregation of cells (Figure 3). The latter phenomenon was not observed by *Langer et al.* [2006], because such a high CO_2 concentration was not used (see also below). The mechanism of aggregation (experiment 5 (Figure 3 and Table 1)) cannot be inferred with confidence from the data

presented here. The unaltered POC production, lowered growth rate, and increased cellular POC content (Table 1) may suggest carbon overconsumption including exsudation of polysaccharides [*Passow*, 2002], which may in turn have increased stickiness and therefore promoted aggregation. A caveat here is the difficulty in obtaining accurate cell densities and so cellular POC content, growth rate, and POC production, in samples containing aggregates. However, scanning electron micrographs revealed stringy organic material (Figure 4) in experiments showing aggregates (experiments 5 and 6 in Table 1). This organic material resembles material which was observed in diatom aggregates showing increased concentrations of extracellular polysaccharides (G. Langer, unpublished data, 2009). It can therefore be speculated that the stringy organic material (Figure 4) consists of polysaccharides, which could have promoted aggregation.

[13] The effect of acid addition on coccolith morphology (Figure 2) was also observed in response to addition of HCO_3^-/CO_3^{2-}, while keeping pH constant (experiment 2 (Figure 5 and Table 1)). This clearly demonstrates that seawater pH is not the parameter of the carbonate system impairing coccolith morphogenesis in *C. leptoporus*, as suggested by *Langer et al.* [2006]. In the following DIC and bicarbonate will be discussed. The difference between the DIC concentration of experiment 1 and that of experiment 3 falls within the range of analytical uncertainty and can safely be regarded as negligible. The difference in coccolith morphology, on the other hand, is very conspicuous. This observation excludes DIC as a possible explanation for hampered coccolithogenesis. This conclusion is also supported when assuming, for arguments sake, that even the tiniest differences in DIC concentration can cause differences in coccolith morphology as will be shown in the following. In experiments 1, 3, 4, and 5 malformations increase while DIC decreases. Hence there is an inverse relationship between DIC and malformations. If we consider experiments 1 and 2 we find an increase in DIC accompanied by an increase in malformations, i.e., a positive correlation. The case of experiments 5 and 6 shows a big difference in DIC, but indistinguishable coccolith morphology. Compared with experiment 3 that means that a small decrease in DIC (experiment 3 and experiment 5) has the same effect on morphology as a big increase in DIC (experiment 3 and experiment 6). The same holds for bicarbonate. Taken together, these observations contradict the hypothesis that DIC is the parameter of the carbonate system affecting coccolithogenesis. Concerning bicarbonate, the following was observed. In experiments 3, 4, and 5 bicarbonate concentration decreases while malformations increase; that is, a negative correlation is observed. In experiment 3 the bicarbonate concentration is higher than in experiment 1, but the percentage of malformations is higher in experiment 3 than in experiment 1; that is, a positive correlation is observed. Therefore the observations also contradict the hypothesis that bicarbonate is the parameter of the carbonate system affecting coccolithogenesis. Please note that this reasoning was employed for arguments sake, i.e., to show that the hypothesis that bicarbonate is the driving force for malformations involves contradictions when tested considering our data set. In practice, the difference between the bicarbonate concentrations of experiment 3 and 4 falls within analytical uncertainty and has therefore to be regarded as negligible. However, doing so one comes to the same conclusion; that is, bicarbonate cannot be the parameter of the carbonate system affecting coccolithogenesis (see discussion of negative correlation in experiments 3, 4, and 5 above).

[14] In the following TA, carbonate ion concentration, and omega calcite will be discussed. In case of TA, it is conspicuous that it decreases in experiments 1, 3, 4, and 5 (higher number corresponds to lower TA (Table 1)), while the percentage of malformed coccoliths increases (Table 1). While this might suggest that decreasing TA causes an increase in malformations, this hypothesis contains contradictions as will be shown in the following. Coccolith morphology in experiment 5 is indistinguishable from coccolith morphology in experiment 6, TA in experiment 6 being more than twice the TA in experiment 5. And, even more importantly, TA in experiment 6 is considerably higher than TA in experiments 1, 3, and 4, the percentage of malformed coccoliths in experiment 6 being higher than in experiments 1, 3, and 4 (Table 1). Therefore TA cannot be the parameter of the carbonate system causing malformations. The same reasoning can be applied with respect to carbonate ion concentration and omega calcite.

[15] To summarize, we have presented compelling evidence that bicarbonate, carbonate ion, DIC, TA, pH, and omega calcite cannot possibly be responsible for hampered coccolith morphogenesis. Thus eliminating the impossible the only parameter of the carbonate system which remains is CO_2. The percentage of malformed coccoliths increases with increasing CO_2 concentration (Table 1). It is therefore concluded that CO_2 is the parameter of the carbonate system causing hampered coccolith

morphogenesis. Comparison of experiments 2 and 3 presents an apparent contradiction to the latter conclusion: the percentage of malformed coccoliths is notably higher in experiment 2 than in experiment 3, despite practically indistinguishable CO_2 concentrations (Table 1). Apparently, the CO_2 effect is somehow enhanced when CO_2 is increased by increasing TA and DIC in concert (experiment 2 in Table 1), as opposed to keeping DIC constant and decreasing TA (experiment 3 in Table 1). This enhancement effect of high DIC can be explained by differences in the carbonate chemistry disequilibrium within the diffusive boundary layer (DBL) of the cell. In the DBL CO_2 is depleted with respect to the bulk seawater due to a CO_2 flux into the cell causing a disequilibrium in the carbonate system. Hence the CO_2 concentration (of the DBL) which the cell actually experiences is lower than that of bulk seawater. Due to the disequilibrium within the DBL the conversion of bicarbonate to CO_2 delivers CO_2. The rate of this conversion increases with increasing bicarbonate concentration. Therefore the resulting CO_2 concentration within the DBL will be higher in experiment 2 than in experiment 3, because both bicarbonate concentration and carbonate ion concentration are considerably higher in experiment 2 (by factors of 4 and 16, respectively). This means that, despite very similar CO_2 concentrations in the bulk seawater, there will be a higher DBL-CO_2 concentration in experiment 2. Therefore the cells experience a higher CO_2 concentration in the latter experiment and it is consequently not surprising that the effect on morphology is more pronounced in experiment 2 than in experiment 3.

[16] Interestingly, CO_2 can be identified as the agent causing aggregation as well. Aggregation was observed in experiments 5 and 6 only (Table 1). Experiment 5 is characterized by the lowest pH while experiment 6 features the second highest pH (Table 1). Therefore pH cannot cause aggregation. DIC and bicarbonate ion concentrations are similar in experiments 1, 3, 4, and 5, while they are notably higher in experiment 6 (Table 1). Since DIC and bicarbonate ion concentrations in the latter experiment, moreover, are considerably lower than those of experiment 2 (Table 1), DIC and bicarbonate can also be ruled out. A similar reasoning and the same conclusion apply to TA, carbonate ion concentration, and omega calcite. Experiment 5 features the lowest values, while experiment 6 is characterized by the second or third highest values (Table 1). Finally, experiment 5 and 6 display the highest and second highest CO_2 concentrations (Table 1). It is therefore concluded that pCO_2 above 1300–1500 μatm causes aggregation of *C. leptoporus* cells. Please note that no data exist between 1300 and 1500 μatm CO_2 (Table 1) and therefore the exact threshold for aggregation remains unknown.

[17] Future research should be concerned with process-level explanations of the described CO_2 effects in *C. leptoporus*. At present it can only be speculated what such explanations might be. As mentioned above, aggregation may involve enhanced production of extracellular polysaccharides. The chain of events leading to impaired coccolith morphogenesis may include acidification of the cytosol due to increased cytosolic CO_2 concentrations (compare also the discussion in *Langer et al.*, 2006).

4. Conclusion

[18] In accordance with an earlier study, it was shown that acidification of seawater leads to an increase in the percentage of malformed coccoliths in *C. leptoporus*. Under pCO_2 higher than 1500 μatm cells form aggregates (>15 cells per aggregate). The data presented here demonstrate that the increase in CO_2, as opposed to, e.g., the decrease in pH, causes both aberrant coccolithogenesis and aggregation of cells.

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