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CALCIFICATION RATES OF EMILIANIA HUXLEYI IN DIFFERENT PH WATERS: A COMPARISON OF METHODS

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In ocean acidification (OA) research, the calcification rate is commonly measured using a variety of methods, so intercomparison of those methods is urgently needed for reliable global analysis of OA effects on organisms. Here, we measured the calcification rates during batch culturing of *Emiliania huxleyi* at three pH (8.2, 8.0 and 7.8) using three methods: budgeting the changes in total alkalinity (TA), Ca ions and particulate inorganic carbon. In this study, these three methods gave similar results: calcification rates decreased with decreasing pH. The rate values obtained from these three methods are comparable. While more intercomparison exercises are mandatory, the present study may suggest that the published data of calcification rates in enclosed incubation systems if derived from the three methods and if properly adopted can be directly intercompared. In addition, the approach of TA budgeting has shown to have higher precision than other two methods owing to its easily achievable better analytical precision, which would be preferable to be adopted for enclosed and low calcification rate systems. It is important to point out that implications of the present study to natural ecosystems are yet to be examined.

1. Introduction

With the rapidly growing research field of ocean acidification (OA), there is an increasing need for standardized protocols in light of the discrepancy commonly occurring to the OA community when for example, doing similar mesocosm/perturbation experiments even by adopting similar experimental protocols. Many of these issues have been highlighted at a number of ocean acidification workshops over the past few years, and have been summarized in the "Guide to Best Practices in Ocean Acidification Research and Data Reporting".[1] A thorough intercomparison for key indexes of OA, and as many others as possible as a continuation of such efforts in order to establish agreed protocols with sound science and technology with wider reaches is clearly needed.

OA is expected to have a highest impact on ocean calcification,[2,3] which has been received wide attention in the community.[4] However, thus far, a variety of methods have been adopted to determine the calcification rates, yet it remains difficult to justify which is the "best" approach for the pelagic systems, spanning from planktonic calcifying organisms, populations and communities.

The short-term gross rates of calcification can be estimated using radioisotope ($NaH^{14}CO_3$ or $^{45}CaCl$) tracer techniques.[5,6] Highly sensitive though, the technique is complicated by the exchange of radioisotope with stable calcium or carbon in the shell.[4] This radioisotope technique has been widely used in laboratory cultures yet it is not easily scaled up for large organisms or communities.

Calcification rates can also be estimated via total alkalinity (TA) depletion or anomaly method.[7,8] This non-destructive method has also been widely used because TA can be very precisely measured. It is also broadly applicable to small (1 L) and short (1–3 h) or large (1000 m^3) and long (days to months) experiments.[7,9] However, the estimation of net calcification through changes in TA has to take into account the changes in nutrient concentrations when their consumption or regeneration is large.[8]

Calcification rates can be moreover estimated via the Ca ions (Ca^{2+}) change method.[10,11] Unlike TA, which is affected by both organic and inorganic carbon production/consumption, the variations of Ca^{2+} in the ocean interior are almost solely controlled by $CaCO_3$ formation and dissolution on day-to-decade time scales.[12] And thus this method is a direct measurement of calcification rate. However, high precision analysis of Ca^{2+} is not routinely acquirable, and thus the method has not been as widely used as the TA method. In addition, this approach is not yet to be used for community calcification rate estimation.

Calcification rates can also be estimated through measuring the particulate calcium carbonate ($CaCO_3$) content.[13,14] However, it remains challenging to reliably determine the $CaCO_3$ concentration in natural environment.

It is known that discrepancies commonly occur to different research groups when adopting single technique and/or when adopting the technique to different experimental environments. More discrepancy may emerge when comparing different methods. It is therefore vitally important to perform intercomparison using the same method among different groups and among different methods to estimate the calcification rates.

In this study, we simulated three levels of pCO_2 (380, 720 and 1,100 ppm) using pH controller and examined the effects of increased pCO_2 on calcification of *Emiliania huxleyi*. Three methods, budgeting the changes in TA, Ca^{2+} and particulate inorganic carbon (PIC), have been used to estimate the net calcification rate of *E. huxleyi* and were intercompared.

2. Material and Methods

2.1. *Incubation condition*

The marine coccolithophorid *E. huxleyi* (calcifying strain CS-369, isolated from Pipe Clay Lagoon, Australia), was cultured in f/25-Si medium at 24°C and 50 µmol photons m^{-2} s^{-1} of photosynthetically active radiation (PAR), in a 14:10 light:dark cycle for 8 days on 26 Jun.–3 Jul. 2009. Cell concentration, pH, Ca^{2+}, TA, PIC and inorganic nutrients were monitored daily during the incubation.

Seawater pCO_2 was adjusted using a pH controller connected to pH electrode.[7] Since pCO_2 in seawater is a function of TA and pH, assuming TA is constant, we can reach a fixed value of pCO_2 through manipulating pH. pH modifications were achieved by bubbling seawater with CO_2-high air (to increase pCO_2). We set three levels of pH (8.2, 8.0 and 7.8), which represent normal (380 ppm), medium (720 ppm) and high (1100 ppm) levels of pCO_2. For the high pCO_2 treatment, the pH controller opened a solenoid valve when pH rose above 7.80, thus bubbling CO_2-high air from a tube to seawater until pH = 7.80 was reached. The pH control mechanism was the same in other two treatments.

2.2. *Carbonate system parameters*

pH was measured with a Corning 350 pH/ion analyzer equipped with an Orion® Ross combination electrode against three NIST-traceable buffers (pH 4.01, 7.00 and 10.01) at a precision of ±0.005. Water samples for TA (pre-filtered slowly with a 0.45 µm PE cartridge membrane) were poisoned with $HgCl_2$-saturated solution and measured by potentiometric titration using Gran procedure.[15] TA determination at a precision of ±2 µmol kg^{-1} was calibrated against the certified reference material from Andrew G. Dickson's lab.[16] pCO_2 and other carbonate parameters were calculated using CO2SYS[17] by pH and TA (Table 1).

Table 1. Mean (SD) values of the carbonate system components for the three cultures of *Emiliania huxleyi* at pH = 8.2, 8.0 and 7.8.

pH_{NBS}	pCO_2 (ppm)	$[HCO_3^-]$ (μmol kg^{-1})	$[CO_3^{2-}]$ (μmol kg^{-1})	$[CO_2]$ (μmol kg^{-1})
8.177	387	1,692	194	11
(0.019)	(38)	(110)	(10)	(1)
7.971	722	1,914	132	21.2
(0.043)	(76)	(57)	(10)	(2)
7.804	1,108	2,043	98	32
(0.044)	(121)	(21)	(10)	(4)

2.3. *Cell and nutrient concentrations*

Cell concentration was analyzed using a Beckman Coulter Multisizer. Inorganic nutrient samples for nitrate (NO_3^-) and phosphate (PO_4^{3-}) analysis were collected with 50 mL polypropylene bottles and were frozen in −20°C until analysis using an AA3 Auto-Analyzer (Bran-Lube, GmbH Co., Germany) according to classical colorimetric methods.[18]

2.4. *Ca^{2+} and PIC*

Ca^{2+} concentration (pre-filtered slowly with a 0.45 µm PE cartridge membrane) was determined using the classic EGTA titration with a Methrom 809 TITRANDO potentiometer.[12] Our replicate measurements revealed a precision of ±10 µmol kg^{-1} in this study. Particle samples were filtered onto pre-combusted (4 h, 500°C) 25 mm Whatmann GF/F filters (pore size 0.7 µm) and then dried at 60°C overnight. Total particulate carbon (without acid fuming) and particulate organic carbon (POC, via acid fuming) were analyzed with a PE-2400 SERIES II CHNS/O analyzer according to the JGOFS protocols.[19] PIC was calculated by the difference. Based on replicate analyses, the precision for the POC determination was <10%.[20]

2.5. *Net calcification rate*

Net calcification rate, also known as net $CaCO_3$ or PIC production rate, was estimated by three independent techniques: budgeting the changes in TA, Ca^{2+} and PIC. According to the equation of calcification,

$$Ca^{2+} + 2HCO_3^- \rightarrow CaCO_3 + CO_2 + H_2O \quad (1)$$

1 mole $CaCO_3$ production consumes 1 mole Ca^{2+} and 2 moles TA. In addition, TA has to be corrected for the effect of primary production, i.e., an uptake of 1 mole $NO_3{}^-$ or $PO_4{}^{3-}$ increases TA by 1 mole.[8] Thus, net calcification rate can be estimated according to the following equations:

$$\text{Net calcification rate} = -0.5 \times (\Delta\text{TA} + \Delta NO_3{}^- + \Delta PO_4{}^{3-})/\Delta t \quad (2)$$

$$\text{Net calcification rate} = -\Delta Ca^{2+}/\Delta t \quad (3)$$

$$\text{Net calcification rate} = \Delta\text{PIC}/\Delta t \quad (4)$$

where ΔTA, $\Delta NO_3{}^-$, $\Delta PO_4{}^{3-}$, ΔCa^{2+} and ΔPIC denote the changes of these parameters. Δt denotes the elapsed time.

3. Results

3.1. *Growth of cells*

The initial cell concentration (0.8×10^5 cells mL^{-1}) was identical in all three treatments. After growth lag phases of 2 days, cells entered exponential growth phases and cell concentrations began to differ greatly among treatments (ANOVA, $p < 0.05$). *Emiliania huxleyi* grew faster in the highest pH treatment. At the end of culturing, cell concentration reached 1.6×10^6 cells mL^{-1} at pH = 8.2, 1.0×10^6 and 5.1×10^5 cells mL^{-1} at pH = 8.0 and 7.8, respectively.

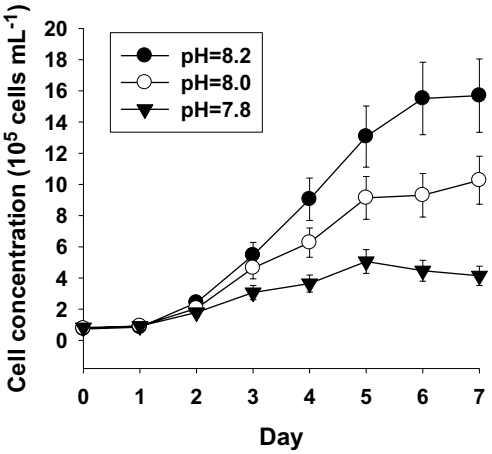

Fig. 1. Effect of pH on growth of *Emiliania huxleyi* in batch cultures. Each data point represents a mean (SD) of three replicates.

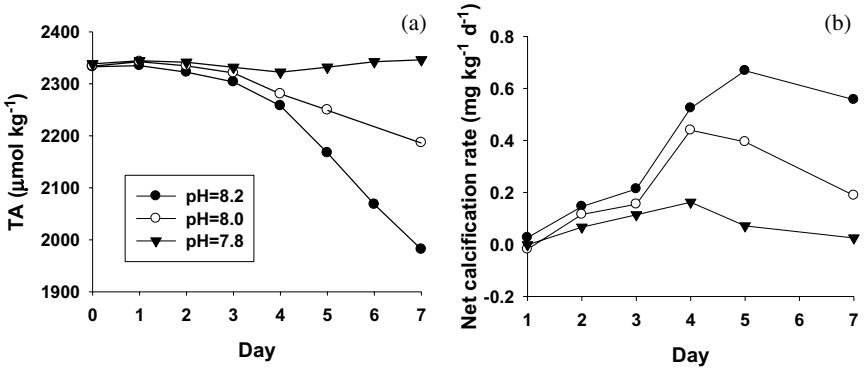

Fig. 2. Effect of pH on (a) total alkalinity (TA) and (b) net calcification rate of *Emiliania huxleyi* measured from Eq. (2) in batch cultures.

3.2. *TA and net calcification rate*

TA was constant at the beginning of the experiment, but decreased sharply from day 2 indicating the onset of calcification by *E. huxleyi*. Decreases of TA ranged from 19 to 65 μmol kg^{-1} between day 2 and day 4. Concomitantly, the discrepancy of TA among different treatments became significant. TA decreased at higher rates until the end of the experiment at pH = 8.2 and pH = 8.0, while TA began to increase slightly from day 4 at pH = 7.8 (Fig. 2a).

According to the TA anomaly calculation (Eq. (2)), the overall net calcification rate ranged 0–0.66 mg kg^{-1} d^{-1}. Calcification started at the onset of exponential growth phases (day 2) and then increased until day 4. From day 3 to day 4, net calcification rates increased rapidly at pH = 8.2 and 8.0 relative to that at pH = 7.8. The highest values were reached on day 4 at pH = 8.0 and 7.8 (0.44 and 0.16 mg kg^{-1} d^{-1}, respectively). But net calcification rate reached its maximum (0.66 mg kg^{-1} d^{-1}) on day 5 at pH = 8.2 (Fig. 2b). Calcification was higher at pH = 8.2 than at pH = 8.0, while it remained low at pH = 7.8. This is consistent with the larger decreases of TA observed at pH = 8.2 and pH = 8.0 compared to pH = 7.8.

3.3. *Ca^{2+} and net $CaCO_3$ production rate*

The Ca^{2+} concentration showed an overall decreasing trend in all treatments, due to the consumption of Ca^{2+} for calcification. Similar to TA, Ca^{2+} concentration decreased faster in higher pH condition (Fig. 3a). From day 2 to day 5, Ca^{2+} concentration declined to 0.12, 0.08 and

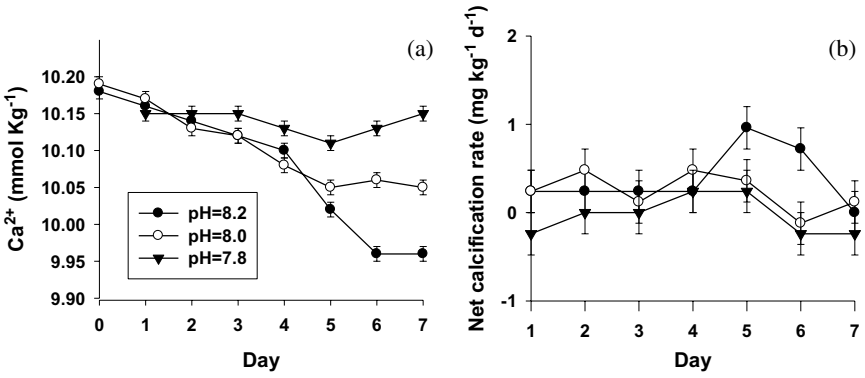

Fig. 3. Effect of pH on (a) Ca ions (Ca^{2+}) and (b) net calcification rate of *Emiliania huxleyi* measured from Eq. (3) in batch cultures. Each data point represents a mean of two replicates.

$0.04\,\mathrm{mmol\,kg^{-1}}$ at pH = 8.2, 8.0 and 7.8, respectively. After day 5, Ca^{2+} concentration stopped dropping at pH = 8.0 and 7.8, but still went down at pH = 8.2.

The difference of net $CaCO_3$ production rates calculated from Ca^{2+} measurements (Eq. (3)) among three treatments was insignificant (ANOVA, $p < 0.05$) before day 4. But on days 5 and 6, $CaCO_3$ production rate was obviously higher at pH = 8.2 than at pH = 8.0 or 7.8 (Fig. 3b). The maximum rate of net calcification was also observed on day 5 at pH = 8.2, which agrees well with the rate estimated from TA budget. Because the average range of duplicate samples is $\pm 0.24\,\mathrm{mg\,kg^{-1}\,d^{-1}}$, the micro-variations of net calcification rates were blurred at pH =8.0 and pH = 7.8.

3.4. *Net PIC production rate*

During the first 2 days, PIC was lower than the detection limit. The highest value observed on day 4 was 0.9, 1.1 and $0.9\,\mathrm{mg\,kg^{-1}}$ at pH = 8.2, 8.0 and 7.8, respectively. From day 4, PIC began to fluctuate. Based on the variations of PIC from day 3 to day 4, the net PIC production rates calculated (using Eq. (4)), were 0.7, 0.7 and $0.4\,\mathrm{mg\,kg^{-1}\,d^{-1}}$ from pH = 8.2 to 7.8.

3.5. *Comparison*

The average net calcification rate during culturing calculated from TA budget was conspicuously lower at pH = 7.8 ($0.07 \pm 0.05\,\mathrm{mg\,kg^{-1}\,d^{-1}}$)

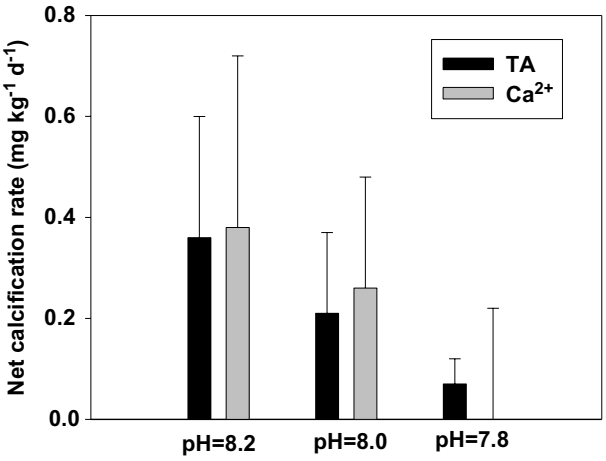

Fig. 4. Mean (SD) of net calcification rate of *Emiliania huxleyi* calculated from TA and Ca^{2+} measurements between day 0 and day 7 in batch cultures at pH = 8.2, 8.0 and 7.8.

than at pH = 8.0 ($0.21 \pm 0.16\,\mathrm{mg\,kg^{-1}\,d^{-1}}$) or at pH = 8.2 ($0.36 \pm 0.24\,\mathrm{mg\,kg^{-1}\,d^{-1}}$). The mean rate of calcification estimated from Ca^{2+} measurement was not significantly different with the rate calculated from TA budget at each pH level (t-test, $p > 0.05$) and also showed lower value with decreasing pH (Fig. 4).

The highest value of net calcification rates estimated from TA, Ca^{2+} and PIC budgets agreed qualitatively with each other and all showed the same declining trends with lowering pH (Table 2). At each pH, the discrepancies of highest net calcification rates calculated from different methods were within their measurement errors, which suggest the comparability of these methods.

During the growth phases of the batch culturing, TA is affected by both photosynthesis and calcification whereas Ca^{2+} and PIC are solely

Table 2. Highest net calcification rates (mg $\mathrm{kg^{-1}\,d^{-1}}$) $\pm$ measurement errors determined by different methods during *Emiliania huxleyi* culturing at pH = 8.2, 8.0 and 7.8. ΔTA, ΔCa^{2+} and ΔPIC denote measurements of calcification by budgeting the changes in total alkalinity, Ca ions and particulate inorganic carbon.

Method	pH 8.2	pH 8.0	pH 7.8
ΔTA	0.66 ± 0.04	0.44 ± 0.04	0.16 ± 0.04
ΔCa^{2+}	1.00 ± 0.24	0.5 ± 0.24	0.24 ± 0.24
ΔPIC	0.7 ± 0.28	0.7 ± 0.28	0.4 ± 0.16

controlled by calcification. It should be more direct to estimate calcification rate via Ca^{2+} or PIC budgeting. However, due to the precision of TA measurement was higher than Ca^{2+} and PIC in this particular study, the measurement error of net calcification rate calculated from TA budget was smaller than the other two methods. Thus, without significant improvement of the measurement precision, using Ca^{2+} and PIC budget methods cannot tell small variations when the net calcification rate is very low during the OA experiment such as the present study.

4. Concluding Remarks

During the batch culturing of *E. huxleyi*, the net calcification rates estimated from TA, Ca^{2+} and PIC budgeting all showed a declining trend with lowering pH. These three methods yielded similar values when calcification rates were high. The present single-incubation study thus suggests that the published data of calcification rates in enclosed incubation systems, if derived from the three methods and if properly adopted, could be directly intercompared. In addition, the approach of TA budgeting has shown to have higher precision than other two methods owing to its easily achievable better analytical precision, which would be preferable to be adopted for enclosed and low calcification rate systems. We must point out however, that the above justification is based upon our single-incubation study and we contend to call more intercomparison exercises for validation. It should also be pointed out that implications of the present study to natural ecosystems are yet to be examined.

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