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Carbonic anhydrase is involved in benthic foraminiferal calcification

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Abstract

Marine calcification is an important component of the global carbon cycle. The mechanism by which some organisms take up inorganic carbon for the production of their shells or skeletons, however, remains only partly known. Although foraminifera are responsible for a large part of the global calcium carbonate production, the process by which they concentrate inorganic carbon is debated. Some evidence suggests that seawater is taken up and participates relatively unaltered in the process of calcification, whereas other results suggest the involvement of transmembrane transport and the activity of enzymes like carbonic anhydrase. Here, we tested whether inorganic carbon uptake relies on the activity of carbonic anhydrase using incubation experiments with the large benthic, symbiont-bearing foraminifer *Amphistegina lessonii*. Calcification rates, determined by the alkalinity anomaly method, showed that inhibition of carbonic anhydrase by acetazolamide (AZ) stopped most of the calcification process. Inhibition of photosynthesis by either 3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU) or by incubating the foraminifera in the dark, also decreased calcification rates, but to a lesser degree than with AZ. Results from this study show that carbonic anhydrase plays a key role in biomineralization of *Amphistegina lessonii* and indicates that calcification of those large benthic foraminifera might, to a certain extent, benefit from ocean acidification.

1 Introduction

Fossil fuel burning and land use changes have been steadily increasing atmospheric CO_2 levels. About 1/3rd of the added carbon has been taken up by the ocean and the resulting increase in seawater dissolved carbon dioxide and associated acidification are lowering the saturation state of sea water and hence likely affects marine calcifiers. Even a modest impact on the production of carbonate shells and skeletons may have important consequences for the global carbon cycle. Foraminifera are responsible for almost 25% of the total marine calcium carbonate production (Langer, 2008) and their response to ongoing acidification is therefore important to predict future marine inorganic carbon cycling. Despite its relevance for future CO_2 scenarios, it is still unclear how increased pCO_2 in seawater will affect foraminiferal calcification. Previous research has shown discrepancies in their results: in some cases a higher pCO_2 increased the growth rate of foraminifera, while in other cases calcification decreased or halted (Haynert et al., 2014; Hikami et al., 2011).

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Addition of CO_2 to sea water not only reduces saturation state but also increases the total dissolved inorganic carbon (DIC) concentration. At surface seawater pH, the dominant DIC species is HCO_3^- and it has been suggested that foraminifera acquire inorganic carbon by actively pumping HCO_3^- from the surrounding seawater to their site of calcification (SOC). In this case, ocean acidification would be detrimental as this shifts the carbonate system from HCO_3^- to CO_2. Alternatively, CO_2 may be the inorganic carbon source of choice for benthic foraminifera, as it diffuses relatively easily through lipid membranes. The latter uptake mechanism would facilitate foraminiferal calcification as ongoing CO_2 dissolution increases total DIC and hence the availability of building blocks for chamber formation. Since this uptake mechanism is crucial for calcification in a rapidly changing ocean and it because it is essentially unknown how foraminifera take up inorganic carbon, it remains difficult to predict the reaction of benthic foraminifera to ongoing environmental change.

It was recently suggested that CO_2 uptake by foraminifera is achieved through proton pumping (Glas et al., 2012; Toyofuku et al., 2017). which increases the pCO_2 directly outside the SOC. The elevated pH at the foraminifers' site of calcification (Bentov et al., 2009; de Nooijer et al., 2009) and reduced pH outside the cell results in a strong inward-outward pCO_2 gradient, enabling inward CO_2 diffusion. If calcification in foraminifera relies on inward CO_2 diffusion, the conversion from HCO_3^- may be a limiting step. This process may be catalyzed by an enzymatic conversion by carbonic anhydrase (CA), which is present in many prokaryotes and virtually all eukaryotes. This enzyme is essential in calcification in many organisms, including corals, sponges and coccolithophores (Bertucci et al., 2013; Medaković, 2000; Müller et al., 2013; Le Roy et al., 2014; Wang et al., 2017). Also for foraminiferal calcification it has been hypothesized that CA is used to enhance carbon uptake. Indirect evidence for such a role in calcification comes from the observed slope between the carbon and oxygen isotopes (Chen et al., 2018), but direct evidence is, however, still missing.

To test whether carbonic anhydrase is involved in biomineralization of benthic foraminifera we incubated calcifying specimens of *Amphistegina lessonii* with acetazolamide (AZ), a membrane impermeable inhibitor of this enzyme. Calcification and respiration were determined by measuring changes in alkalinity and DIC of the incubated seawater over the course of the experiment. An additional experiment was conducted in parallel to test whether CA is directly involved in calcification or that the effect is indirect. The latter would imply that CA drives photosynthesis by the symbionts and that observed effects would be due to reduced photosynthesis impairing calcification through reduced energy transfer from the symbionts to the foraminifer.

2 Material and methods

2.1 Foraminifera and incubations

Surface sediments were collected from the Indo-Pacific Coral reef aquarium in Burgers' Zoo (Arnhem, the Netherlands; Ernst et al., 2011). The sediments were kept at 24 ºC, with a day/night cycle of 12h/12h. Living specimens of *Amphistegina lessonii* showing a dark cytoplasm and pseudopodial activity were manually selected, using a fine brush under a stereomicroscope and transferred to Petri dishes. They were fed with freeze-dried *Dunaliella salina* and incubated in North Atlantic seawater in which calcein was added at a final concentration of 5 mg/L. After a week, viable specimens where collected and divided over eight experimental conditions, each of them consisting of three groups. Each group consisted of 40-60 specimens with a similar size distribution. Foraminifera were placed in air-tight glass vials of 80 ml (24°C, 12h day-light cycle) for 5 days.

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In the first experiment, the impact of acetazolamide (AZ) on calcification was tested. A stock solution was prepared by dissolving acetazolamide (Sigma-Aldrich) in dimethyl sulfoxide at a final concentration of 90 mM. The AZ stock solution was diluted with seawater from North Atlantic to achieve AZ concentrations of 4, 8 and 16 µM, which were used to incubate the foraminifera in. In a second experiment, inhibition of photosynthesis was tested by 1) addition of 3-(3,4-Dichlorophenyl)-1,1-dimethylure (DCMU) and 2) darkness. DCMU was added to seawater at a final concentration of 6 µM, whereas covering the vials with aluminum foil prevented light-dependent reaction and hence photosynthesis in a second set of incubations (Fig. 1).

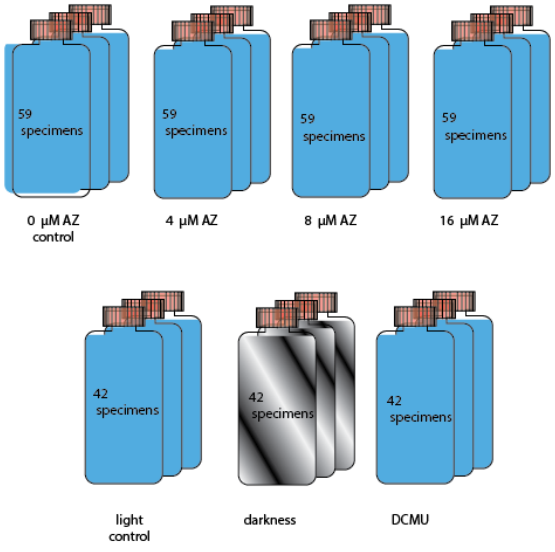

Figure 1: ***59 specimens were placed in one culture vial, with three replicate vials for each concentration of acetazolamide (upper row). Similarly, 42 specimens were incubated under light, in the dark and with the inhibitor DCMU (lower row).***

2.2 Alkalinity, DIC and nutrient analysis

To quantify calcification and respiration, total alkalinity (TA) and the concentration of dissolved inorganic carbon [DIC] were determined at the beginning and end of every incubation. Total alkalinity was analyzed immediately at the end of each experiment, whereas subsamples to determine nutrient concentrations and DIC analyses were stored at -20°C (nutrients) and 4°C (DIC). The samples for DIC analyses were poisoned with mercury chloride (DIC) until analysis. These samples first passed a 0.2 µm syringe filter.

Alkalinity measurements were performed using an Automated Spectrophotometric Alkalinity System (ASAS), as described by (Liu et al., 2015). Briefly, 60 mL of seawater are placed in a borosilicate vial and automatically titrated with a solution of 0.1 M HCl. Before the start of the titration, 45 microliters of bromocresol purple (10 mmol/L) was added to the seawater and pH evolution is followed by spectrophotometry. Certified reference material (CRM; Dr. Dickson, Scripps Institution of Oceanography) was analyzed at the beginning of every series (5-10 samples) of measurements. Reproducibility of the obtained TA was ~3 µmol/kg (SD), based on 50 measurements of untreated seawater.

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Nutrient samples were analysed on a QuAAtro continuous flow analyzer (SEAL Analytical, GmbH, Norderstedt, Germany) following GO-SHIP protocol (Hydes et al., 2010). DIC was measured on an autoanalyzer TRAACS 800 spectrophotometric system as described in Stoll et al. (2001).

2.3 Calcification rate

Changes in DIC and alkalinity between start and end of the experiments were used to calculate the net respiration and calcification (Fig. 2). Total measured alkalinity is defined as the contribution of the following anions:

$$T_{Ameasured} = [HCO_3^-]_T + 2[CO_3^{2-}]_T + [OH^-]_T + 3[PO_4^{3-}]_T + [HPO_4^{2-}]_T + [NO_3^-] - [H^+]_T - [NO_4^+] \quad (1)$$

Concentrations of boron and silicon were neglected as the first one is constant the second present at a low abundance. In order to account for the alkalinity change related to the inorganic carbon system only, we subtracted the combined concentrations of the nutrients from the measured alkalinity so that the observed change in alkalinity over time is defined as:

$$T_A = [HCO_3^-]_T + 2[CO_3^{2-}]_T + [OH^-]_T - [H^+]_T \quad (2)$$

$Resp_{net}$ is defined as the difference between respiration and photosynthesis. Here, we consider the respiration of the holobiont (foraminifera and its symbionts), which is calculated by:

$$Resp_{net} = delta\ DIC - delta\ TA/2 \quad (3)$$

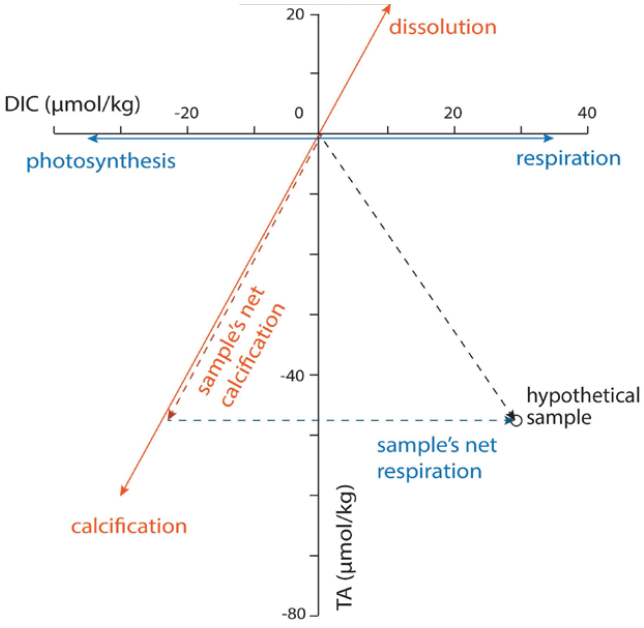

Figure 2: Calcification and net respiration of foraminifera deduced from changes in DIC and total alkalinity over time.

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3 Results

3.1 Carbonic anhydrase inhibition

Without acetazolamide, T_A decreased on average by 53 µmol.kg^{-1} and DIC by 38 µmol.L^{-1} during the incubation (table 1). This corresponds to 2.74 g/L of precipitated calcite. Contrastingly, when the seawater contained acetazolamide (even at the lowest concentration of 4µM), alkalinity and DIC did not change or decreased only marginally during the incubation (less than 0.4 g/L of calcite precipitated). When comparing the changes in T_A and DIC between treatments, calcification is minimized by the AZ and net respiration slightly increases (Fig. 3). The concentration of AZ has no discernible effect on the magnitude of changes in calcification/ respiration.

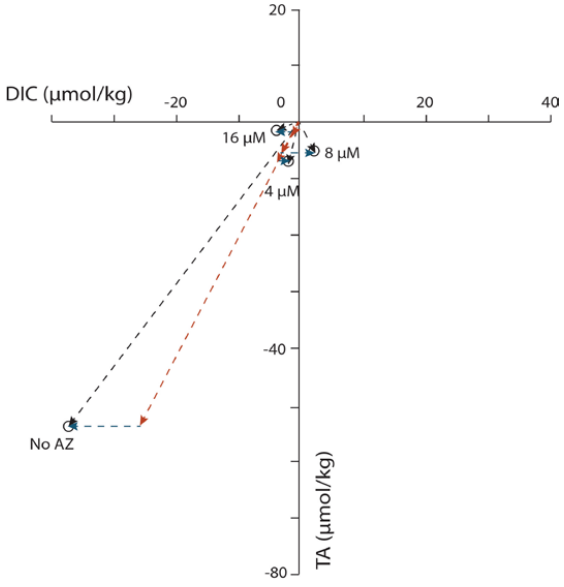

Figure 3: Changes in total alkalinity versus DIC for all concentrations of acetazolamide (AZ) used. Every circle represents the average change in DIC-TA for one triplicate of incubations.

[AZ] (µM)	Initial Ta	Δ Ta	Initial DIC	Δ DIC
0	2283.9	53.2± 8.3	2109.7	37.9 ± 9.0
4	2285	6.9 ± 1.2	2105.4	1.6 ±2.1
8	2285.4	5.1 ± 1.2	2105.3	-2.7 ±6.7
16	2292	1.6 ± 3.6	2108.8	3.4 ±5.7

Table 1 : Total alkalinity and DIC values measured in the vials

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3.2 Photosynthesis inhibition

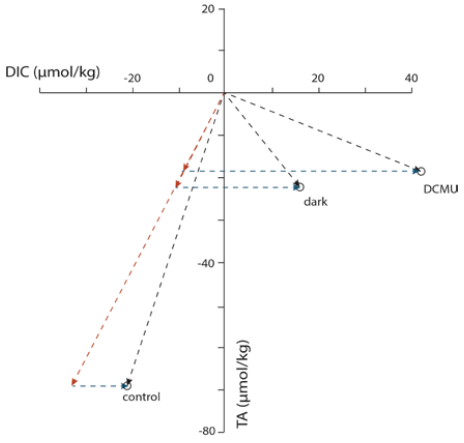

Figure 4: Changes in total alkalinity versus that in DIC for incubations in light-dark alternation (control), in the dark and with the photosynthetic inhibitor DCMU. Every circle represents. the average change in TA and DIC between the initial and the final values for each triplicate. Arrows show the calcification (red) and net respiration (blue) effects.

Vial	Initial Ta	Δ Ta	Initial DIC	Δ DIC
control	2280.1	69.7±7.3	2115	21.0±9.0
DCMU	2286	22.3±9.3	2090.7	-42.2±13.8
dark	2280.1	18.6±5.6	2115	-16.3±5.2

Table 2: Total alkalinity and DIC change for every triplicate. Confidence interval: 1 STD (taking biological variability into account)

When photosynthesis was not impaired (light control), alkalinity decreased within the vials by 69.7 µmol·L^{-1} and DIC increased by 21 µmol·L^{-1} (table 1). Given the relative standard deviations, this is similar to the changes in TA and DIC in the control vials for the AZ-experiments. These changes correspond to approximatively 3.75 g.L^{-1} of precipitated calcite. In contrast, when foraminifera were cultivated in the dark or in presence of the photosynthesis inhibitor DCMU, DIC increased by 37.7 µmol·L^{-1} whereas the total alkalinity decrease was only 22.8 µmol·L^{-1}, which corresponds to less than a third of the amount of calcite precipitated when photosynthesis was not hampered (Fig. 4).

4. Discussion

4.1 Growth rates and the effect of AZ

In the control experiments (incubations with unaltered seawater), foraminiferal calcification resulted in a decrease in alkalinity of the culture media by approximately 65 µmol·L^{-1} over a period of 5 days (table 1). On average, this equals a growth rate of 1.0 µg·$Ind.^{-1}$·day^{-1}, which is low when compared to some previously reported rates (~6-60 µg·$Ind.^{-1}$·day^{-1}; (Evans et al., 2018; Glas et al., 2012; Keul et al., 2013). These studies, however, all used different species than the one incubated here. The only previous study using *Amphistegina* spp. (Segev and Erez, 2006) reported growth rates similar to those observed here (0.53-1.0 µg·$Ind.^{-1}$·day^{-1}), based on changes in dry weight. The similarity in growth rates despite the different approaches used, suggests that the alkalinity anomaly method reflects growth rates accurately.

Addition of AZ lowered calcification rates by approximately 20 times (Fig 2), while increasing net respiration. The concentration of the inhibitor (4-16 µM) did not affect the magnitude by which net calcification decreased,

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nor does it appear to affect the increase in net respiration (Fig. 3). The inhibition of calcification caused by AZ suggests that carbonic anhydrase plays a crucial role in foraminifera biomineralization. With the inhibitor present foraminifera produced little to no calcite (figure 3), indicating that either biomineralization relies on CA, or is negatively impacted through an effect of CA on photosynthesis. Whether calcification depends directly on extracellular carbonic anhydrase (eCA) or that calcification depends on photosynthesis and thereby indirectly on CA, can be inferred from comparing the two sets of experiments (Fig. 1).

4.2 Effect of photosynthesis on calcification

The inhibition of photosynthesis with DCMU and darkness decreases calcification comparably (Fig. 3). Simultaneously, net respiration increases after addition of DCMU, as well as by blocking light (Fig. 4). The similarity in the effect of darkness and DCMU indicates that photosynthesis has an effect on calcification. It was previously suggested that light, irrespective of photosynthesis, enhances calcification in foraminifera (Erez, 2003). Since the latter study used the planktonic, low-Mg calcite *Globigerinoides sacculifer*, the discrepancy between results may be caused by differences in the process involved in calcification between these species.

Foraminiferal calcification and endosymbiont photosynthesis both require inorganic carbon. Therefore, it seems reasonable to suggest that those two mechanisms are competing with each other for inorganic carbon, as was shown by (Ter Kuile et al., (1989b, 1989a). However, our results show that preventing photosynthesis by the symbionts actually decreases foraminiferal calcification. This implies that benefits from photosynthesis overcomes an eventual competition with calcification, which is in agreement with results from Duguay (1983) and Hallock (1981) who showed that both calcium- and inorganic carbon uptake into the cell is enhanced by light.

A positive effect of photosynthesis on calcification has been observed previously for other marine calcifyers as well. For example, in coccolithophores, decreasing CO_2 can hamper calcification through reduced photosynthesis (Mackinder et al., 2010). This can be explained by production of organic molecules linked to photosynthesis, which act as organic templates for calcification. We here hypothesize that a similar effect may explain decreased calcification in foraminifera as a consequence of inhibited photosynthesis (Fig. 3). If so, the type of organic molecules produced by the foraminifer's endosymbionts and their fluxes will need to be assessed to test the extent of the dependency of calcification on photosynthesis. However, it has been shown that symbiotic dinoflagellates and zooxanthellae can trigger the activity of carbonic anhydrase in their host organisms (giants clams and sea anemones) (Leggat et al., 2003; Weis, 1991; Weis and Reynolds, 2002; Yellowlees et al., 2008), thereby explaining how photosynthesis enhances calcification.

4.3 Role of CA in calcification

In calcifyers other than foraminifera, carbonic anhydrase plays a direct role in calcification. In for example, giant clams (Chew et al., 2019), corals, gastropods (Le Roy et al., 2012) and oysters (Wang et al., 2017), CA helps to concentrate inorganic carbon in the fluid from which calcium carbonate precipitates. In scleractinian corals, CA promotes conversion of metabolic CO_2 into bicarbonate after the carbon dioxide diffused into the sub-calicoblastic space. Although the inorganic carbon would take the same route in absence of CA, the hydration of CO_2 is relatively slow and ion fluxes and calcification rates would be a fraction what they are with the catalytic activity of CA. This role of CA fits with the localization of (membrane-bound) CA observed at the walls of the calicoblastic

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cells by immunolabelling (Moya et al., 2008). In addition, by facilitating an inward flux of inorganic carbon, involvement of CA can explain the co-variation of oxygen and carbon isotopes in coral aragonite (Chen et al., 2018; Uchikawa and Zeebe, 2012). Also by the reversed process, the dissolution of $CaCO_3$ by excavating sponges, CA plays an important role, especially in the dark where increased CA activity promotes outward diffusion of CO_2 resulting from $CaCO_3$ dissolution (Webb et al., in prep).

In larger benthic foraminifera, CA likely plays different roles: it helps concentrating CO_2 by the symbionts and aids foraminiferal calcification. It still remains to be investigated which molecular types of CA are involved and where they are located precisely within the larger benthic foraminifera. Analogous to other calcifying organisms and based on existing models of foraminiferal calcification, we hypothesize that CA helps to convert HCO_3^- into CO_2 directly outside the calcifying chamber. This would help to further increase the pCO_2 outside the foraminifer in addition to the shift in inorganic carbon chemistry resulting from active proton pumping and subsequent low pH (Glas et al., 2012; de Nooijer et al., 2009; Toyofuku et al., 2017). Although not directly targeted by our experimental approach, as the inhibitor we used is membrane impermeable, it is likely that a form of CA within the calcifying fluid increases the rate by which the diffused CO_2 is converted into bicarbonate.
The involvement of CA in calcification may explain why foraminifera can be relatively resilient to ocean acidification. If they rely on CA for conversion of HCO_3^- to CO_2 and take up inorganic carbon by diffusion of CO_2, additional dissolved atmospheric CO_2 may be beneficial for calcification in foraminifera. If they exclusively rely on bicarbonate ions, a reduction in pH would lower the $[HCO_3^-]$ and thereby hamper calcification. Manipulation of the inorganic carbon speciation in relation to calcification and the aid of enzymes therein, will allow predicting rates of calcification as a function of ongoing ocean acidification.

5 Conclusions

The alkalinity anomaly method allowed us to quantify growth rates in incubation experiments, equalling addition of 1 µg/individual/day. Calcification and photosynthesis in the benthic foraminifer *Amphistegina lessonii* and its symbionts both depend on carbonic anhydrase (CA) as shown after inhibition by acetazolamide (AZ). Since the inhibitor is membrane-impermeable, the CA may well be localized at the outside of the foraminifer's cell membrane. Our results also show that inhibiting photosynthesis by DCMU or incubation in darkness reduce calcification similarly. This suggests that not light, but photosynthesis itself promotes calcification. We also suggest that CA plays a role in concentrating inorganic carbon for calcification, possibly by promoting conversion of bicarbonate into carbon dioxide outside the foraminifer.

Data availability

The data on which this publication is based can be found through the following DOI: 10.4121/uuid:afcdcdc1-2591-4822-bade-806119cdd724

Authors contribution:
SdG and LJdN designed the experiment and SdG carried it out. SdG and AEW analysed the seawater inorganic chemistry. SdG and LJdN analysed the data and prepared the manuscript with contributions from all co-authors.

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248 **Competing interests**

249 The authors declare they have no conflict of interest

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