THE POTENTIAL ROLE OF INTERNAL PH MANIPULATION BY ACTIVE PROTON PUMPING IN FORAMINIFERA DURING BIOMINERALIZATION

MSc thesis

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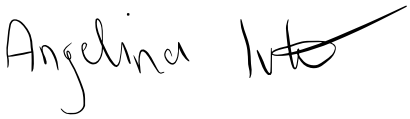
Signature: 

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Abstract

Calcifying foraminifera are one of the major marine calcifiers in open oceans and they are affected by ocean acidification. However, their response to acidified seawater is not according to inorganic precipitation but a biological regulation takes place during biomineralization. Two widely accepted models exist to explain the biological control during calcification, both supporting internal pH regulation. To predict how these calcifying organisms will be affected by increased pCO_2 levels and therefore, a lowered seawater pH, culture experiments investigating calcification rates are necessary. Here we present results from a culture experiment investigating the response of two benthic symbiont-bearing calcifying foraminifera under a range of four pCO_2 concentrations (400, 700, 1000 and 2200 ppm) projected for the future. The greatest change in total alkalinity caused by calcification was observed at a pCO_2 concentration of 700 ppm, while it was significantly lower at 1000 and 2200 ppm, indicating less successful biomineralization under very high pCO_2 concentrations. Calcification rates suggest species specific responses to ocean acidification with *H. depressa* performing worse under the 2200 ppm treatment than *A. lessonii.* The different responses of the foraminifera between varying pCO_2 treatments and between the two different species suggest that at least some foraminifera will be able to cope with increasing atmospheric CO_2 concentrations.

Keywords

Foraminifera

Ocean acidification

Biomineralization

Calcification rates

Alkalinity anomaly technique

Introduction

Foraminifera are unicellular eukaryotic organisms ranked in the SAR (Stramenopiles, Alveolates and Rhizaria) group (Parfrey et al., 2011, Adl et al., 2005) and they are often associated with test formation. These tests are constructed from a variety of materials such as organic constituents, sediment grains or $CaCO_3$ (Gupta, 1999). Calcareous foraminifera are important marine calcifiers that can contribute up to 50% of the biogenic calcite in open oceans (Schiebel, 2002). Furthermore, their $CaCO_3$ tests are of particular interest for paleoclimatic reconstructions, as they provide information on past seawater chemistry and therefore, environmental parameters.

1. Ocean acidification and $CaCO_3$

Since the industrial revolution, atmospheric CO_2 values are continually rising from pre-industrial values of 280ppm to a present value of over 400ppm (IPCC, 2013). The ocean is one of the largest CO_2 sinks on earth due to a continuous exchange between the atmosphere and the sea. Increased dissolved inorganic carbon (=DIC; CO_2, H_2CO_3 & HCO_3^-) in surface oceans results in a change of oceanic carbon chemistry. As more carbon dioxide enters the sea, it reacts with water to carbonic acid (H_2CO_3), which dissociates to bicarbonate (HCO_3^-) and protons (H^+). Sequentially, the protons react with the available carbonate ions (CO_3^{2-}) to form more bicarbonate ions and therefore, carbonate ion concentrations decrease (see Fig. 3 in Ridgwell and Zeebe (2005)) (Zeebe and Wolf-Gladrow, 2001, Ridgwell and Zeebe, 2005, Hoegh-Guldberg et al., 2007, Doney et al., 2009). The uptake of carbon dioxide in the ocean, which results in a decrease of surface ocean pH ($pH= -\log_{10}[H^+]$) and in a shift of carbonate speciation, is called ocean acidification.

It is widely believed, that precipitation of calcium carbonate depends on the saturation state, which is defined as $\Omega = [Ca^{2+}] * [CO_3^{2-}]/K_{sp}$ (with $K_{sp} = [Ca^{2+}]_{sat} * [CO_3^{2-}]_{sat}$) and therefore, on the concentration of calcium- and carbonate-ions (Zeebe and Wolf-Gladrow, 2001). The solubility product (K_{sp}) is dependent on *in situ* temperature, salinity, pressure and the mineral phase (Zeebe and Wolf-Gladrow, 2001). While the temperature effect is small, the solubility of $CaCO_3$ increases with pressure and therefore, with depth (Zeebe and Wolf-Gladrow, 2001). Apart from this effect due to the solubility product, the $CaCO_3$ saturation state in the open ocean is mainly controlled by the carbonate ion concentration, because the variation in concentration of calcium is rather small (it is related to salinity) (Zeebe and Wolf-

Gladrow, 2001, Mucci, 1983). As with ongoing ocean acidification, carbonate ion concentrations are decreasing, calcite and aragonite precipitation would therefore be hampered under future scenarios (Zeebe and Wolf-Gladrow, 2001).

2. The biological control

In experiments with increased CO_2 concentrations, different responses of various organisms were found (Doney et al., 2009). Some reduced their calcification and growth rates at higher CO_2 concentrations, while others were found to be unaffected, and in some cases, experienced increased calcification (see Fig. 4 in Doney et al. (2009) for comparison). Foraminifera have been found to display varying responses to acidified seawater. For the miliolid species *Margionopora kudakajimensis* a non-linear response to elevated CO_2 concentrations was found, with no significant differences between a seawater pH treatment of 7.9 and 8.2 (modern sea-water pH), but a decline in calcification at a pH of 7.7 (Kuroyanagi et al., 2009). For other calcifying foraminifera from the Rotaliid group with diatom symbionts, such as *Baculogypsina sphaerulata* and *Amphisorus hemprichii*, increased calcification was observed at pCO_2 levels of 580 and 770 ppm, while calcification rates decreased under even more elevated pCO_2 concentrations of 970 ppm (Fujita et al., 2011). Therefore, foraminiferal tests are not built purely according to inorganic precipitation, but several studies have shown a biological control on the microenvironment during biomineralization. This biological control leads to a disequilibrium between the seawater elemental composition and the elements incorporated into the foraminiferal shells, which are termed “the vital effect” (Urey et al., 1951, Weiner and Dove, 2003).

3. Foraminiferal biomineralization

To understand the effects of ocean acidification on foraminifera and to improve proxy calibrations, an understanding of the underlying mechanisms of chamber formation is necessary. To precipitate their $CaCO_3$ shells, foraminifera have to elevate the carbonate-concentrations at the site of calcification (SOC), because the predominant carbonate species in modern seawater is bicarbonate (see Fig. 3 in Ridgwell and Zeebe (2005)) (Evans et al., 2018, de Nooijer et al., 2014). Furthermore, the mechanism should allow for the reduction of magnesium-ions and concentrate calcium-ions at the SOC, as magnesium is an inhibitor during $CaCO_3$ precipitation (de Nooijer et al., 2014).

Two opposing, but not mutually exclusive, biomineralisation models for foraminiferal calcification are widely accepted in the foraminiferal community: The seawater vacuolization model and the trans-membrane transport model (see Evans et al., 2018, and de Nooijer et al., 2014 for summary). While the former hypothesizes the internal storage and accumulation of ions necessary for calcification in seawater vacuoles (e.g.: Bentov et al., 2009), the latter suggests an active transport of selected ions through transmembrane pumps and channels (e.g.: Toyofuku et al., 2017).

Recently, Toyofuku et al. (2017) suggested a biomineralisation mechanism for foraminifera, which combines the ability to pump calcium to, and simultaneously concentrate carbonate at the SOC. This model suggests that the benthic foraminifera *Ammonia sp.* is able to control the internal pH at the SOC which enables it to transform diffused CO_2 into the necessary carbonate-ions for calcification (de Nooijer et al., 2014, de Nooijer et al., 2009, Glas et al., 2012, Toyofuku et al., 2017). Toyofuku et al. (2017) showed that this foraminifera actively pumps protons out of the protoplasm during chamber formation using a V-type H^+ ATP-ase. As protons are being expelled, the pH in the close vicinity of the specimen decreases, which leads to a shift in the carbonate system with increased CO_2 concentrations, thus enhancing diffusion of CO_2 into the protoplasm (Toyofuku et al., 2017). There, the carbon dioxide is being transformed to carbonate (due to higher pH), which is ultimately used for calcification (see Fig. 3 in Toyofuku et al. (2017)) (Toyofuku et al., 2017).

This implies that the chamber formation in foraminifera is not mainly dependent on the carbonate concentrations in the seawater but increased total DIC might enhance calcification (Toyofuku et al., 2017). Transmembrane transport models coupling calcium uptake to proton pumping have also been established in corals (Sinclair and Risk, 2006, McConnaughey and Whelan, 1997) and cyanobacteria (Ogawa and Kaplan, 1987) and Zeebe et al. (2008) suggest that outward proton pumping is more energy-efficient than magnesium removal at the site of calcification (for a summary see de Nooijer et al., 2014).

4. Research aim

Internal pH elevation by outward proton pumping is an energy consuming mechanism and it would be more difficult for foraminifera to elevate the internal pH in acidified seawater (Bentov et al., 2009). However, the lowered pH in the close vicinity of the foraminiferal shell would, in turn, lead to a higher CO_2 availability, enhancing carbon dioxide diffusion to the site

of calcification. To conclude, there is a need to find the balance, herein called tipping point, between gains of increased DIC availability and costs for proton pumping in more acidic oceans. Knowing how the calcification rates are affected by increased pCO_2 is essential for predicting how foraminifera will respond to climate change.

In this study, a culture experiment with the two benthic species *Amphistegina lessonii* (d´Orbigny, 1843) and *Heterostegina depressa* (d'Orbigny, 1826) was conducted. Both are hyaline, low Mg-calcite benthic foraminifera from the *Rotaliid* group (Gupta, 1999), bearing diatom photosymbionts. They were exposed to four different pCO_2 levels (400, 700, 1000 and 2200 ppm) under controlled conditions and the amount of precipitated $CaCO_3$ was determined using the alkalinity anomaly technique (Smith and Key, 1975). The data allows to determine the tipping point where energy consumption surpasses energy gain under elevated CO_2 concentrations by comparing the obtained calcification rates. This enables us to predict how *A. lessonii* and *H. depressa* will react to ocean acidification. Furthermore, insight about the validity of different biomineralization models was gained.

Material & Methods

1. Collection

Samples were collected in August 2018 from the tropical reef aquarium at the Burger´s Zoo in Arnhem and brought to the royal Netherlands Institute for Sea Research (NIOZ). The high densities of benthic foraminifera found in this artificial habitat were described by Ernst et al. (2011), with *Heterostegina depressa* being the most abundant species. The sediments we received, were split and transferred to two small aerated aquaria at ambient laboratory temperatures and with no additional light source apart from the ambient light. From this stock, foraminifera were picked from the sides of the aquaria (Fig. 1) and additionally they were carefully detached from the sediment with a brush. The most commonly found species in our sediment was *Amphistegina lessonii*, followed by *Heterostegina depressa* and some miliolid species, such as *Quinqueloculina sp*.

Figure 1. Aquarium with sediments (mainly coral debris) from Burger´s Zoo. Foraminifera are well visible as little dots on the walls of the aquarium.

Isolated living individuals of *A. lessonii* and *H. depressa*, recognized by their brown cytoplasm and pseudopodial activity, were transferred to petridishes with filtered (0.2 μm) Atlantic seawater, fed with freeze-dried *Dunaliella salina* and kept in incubators at 24°C. Feeding and water exchange were conducted once a week while they were kept in the incubators.

One week before the culture experiment started, the foraminifera were transferred to 50 ml Falcon® tissue culture flasks with a vented cap: 50 specimen of *A. lessonii* and 25 specimen of *H. depressa* were used per culture flask. The Falcon® bottles were filled with 50ml of filtered Atlantic seawater which was spiked with the fluorescent dye Calcein (5mg/L seawater) (Fig. 2). The reason for spiking the water is that the foraminifera building new chambers during the week will incorporate the Calcein into their $CaCO_3$ shells (Bernhard et al., 2004) thereby providing the possibility to detect which chambers were built during that week with a fluorescent microscope. The specimens were kept in those flasks with the Calcein water in the incubator at 23°C for a week. After the incubation, the Calcein-spiked seawater was exchanged for Atlantic filtered seawater without Calcein and foraminifera were fed and transferred to the culture experiment.

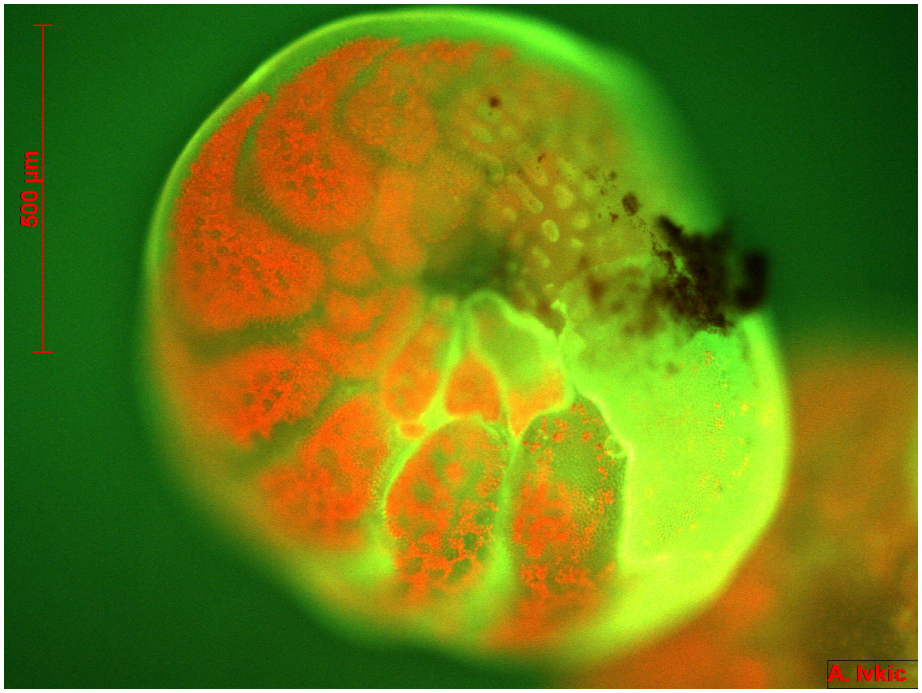

Figure 2. A. lessonii during the incubation with Calcein. The three chambers built during the incubation with Calcein are fluorescent green. Symbionts are visible in red. The last chamber was probably built quite recently, thus not yet filled with symbionts although it is already fluorescent green.

2. Culture set-up

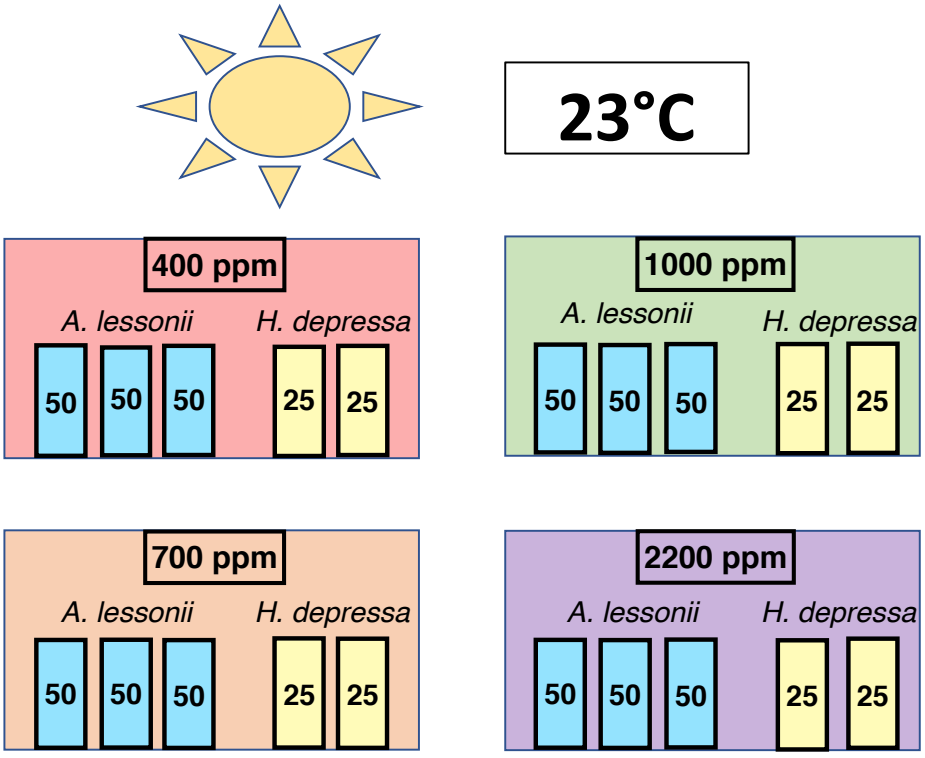

Figure 3. Culture set up. Three replicates with 50 A. lessonii each and two replicates with 25 H. depressa each were kept in four different CO_2 conditions of 400, 700, 1000 and 2200 ppm with constant light and temperature.

The set-up of the culture experiment consisted of four chambers with a controlled atmosphere. This is achieved by a complex apparatus built by senior research assistant Bob Koster at the NIOZ (Fig. 4, Fig. 5). The simplified explanation of the set-up is as follows: Outside air is compressed and led into a bottle filled with soda lime (Soda lime with indicator, AnalaR NORMAPUR® analytical reagent, VWR Chemicals). The soda lime binds any present CO_2 and therefore, the air is CO_2-free after this step. This air is being mixed with pure CO_2 in a certain concentration (controlled by a valve) and the artificially CO_2-enriched air is being led first into an air-tight bottle filled with MiliQ water, which ensures that the air is humidified, and from there into an enclosed chamber with a CO_2 sensor (Vaisala GMD20). The CO_2 sensor measures the concentration of carbon-dioxide within the chamber and communicates with the valve controlling the CO_2 input. After a target-CO_2 concentration is being set, the valve and the sensor communicate in a way to reach the desired carbon-dioxide concentration after a few minutes within the chambers. Each of the four chambers can target a certain atmospheric CO_2 concentration, which were chosen with 400 ppm, 700 ppm, 1000 ppm and 2200 ppm ± 2% CO_2 for this experiment.

The main advantage of this approach compared to a pH manipulation experiment with acid/base titrations is that it allows to calculate the precipitated $CaCO_3$ with the alkalinity anomaly technique (Smith and Key, 1975). The alkalinity anomaly technique relies on the assumption that the total alkalinity is decreased by two moles, when one mole of CaCO3 is precipitated (see Fig. 1.6.24 in Zeebe and Wolf-Gladrow (2001)) (Smith and Key, 1975, Langdon et al., 2010, Zeebe and Wolf-Gladrow, 2001). The addition and subtraction of CO_2 does not affect the total alkalinity (TA), which can be simplified as TA$\approx [\mathrm{HCO_3^-}] + 2[\mathrm{CO_3^{2-}}] + [\mathrm{B(OH)_4^-}] + [\mathrm{OH^-}] - [\mathrm{H^+}]$ (see Zeebe and Wolf-Gladrow (2001)). When CO_2 is added to seawater under current pH (around 8.2), it reacts with water to form bicarbonate and protons: $\mathrm{CO_2 + H_2O \leftrightarrow HCO_3^- + H^+}$; therefore, not changing net TA. However, when acid or base is added to seawater, the TA is influenced. Here an example: If a strong acid, like HCl is added to seawater it dissociates to Cl^- and H^+, which then changes TA as the concentration of protons changes.

To summarize, if ocean acidification experiments are being conducted with CO_2 manipulation instead of acid/base titration, the production of $CaCO_3$ in seawater can be evaluated by measuring total alkalinity before and after an incubation period with foraminifera. In this experiment, the produced $CaCO_3$ of the two species was estimated based on those

measurements. Additionally, the data was corrected for salinity and nutrient samples (NO_3^-, NO_2, PO_4^{2-} and NH_4^+) were taken, as these nutrients can have an influence on TA by adding or removing the equivalent of acid from the seawater (Jacques and Pilson, 1980). Apart from the methodological advantages of the CO_2 manipulation, it also represents a more realistic response of the ocean to anthropogenic increased CO_2 (Fujita et al., 2011).

The whole set up is situated in a climate-controlled chamber without any ambient light and with controlled temperatures. Light was artificially introduced by lamps with a 12h/12h light-/darkcycle to imitate day and night. The light intensity received within the chambers was measured with a light meter and was held constant during the "day-conditions" (lightcycle) in all four chambers at 240µmol/m^2/sec. The temperature of the room was set at 23°C and the temperature within the chambers was monitored additionally with a temperature logger (Traceable Logger Trac, Maxi Thermal). A varying temperature was noticed within the chambers between max. 24°C during light-conditions and min. 21°C during dark conditions. Although varying between "day" and "night", the variability was constant and on average the temperature within the chambers was 22.78°C.

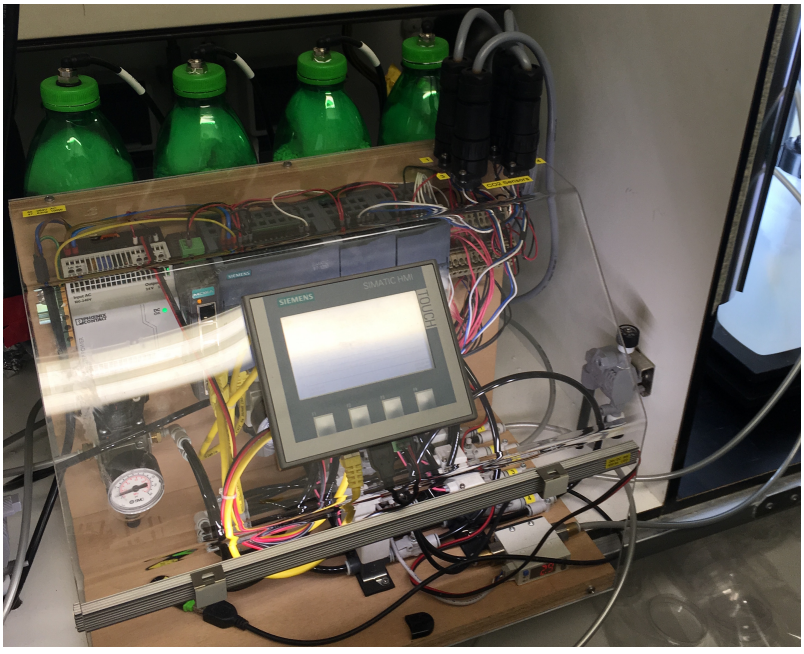

Figure 4. Control panel for culture experiment set-up. The targeted carbon-dioxide concentrations are set here. In the back green bottles are visible: This is where the CO_2 is being scrubbed out of the compressed air.

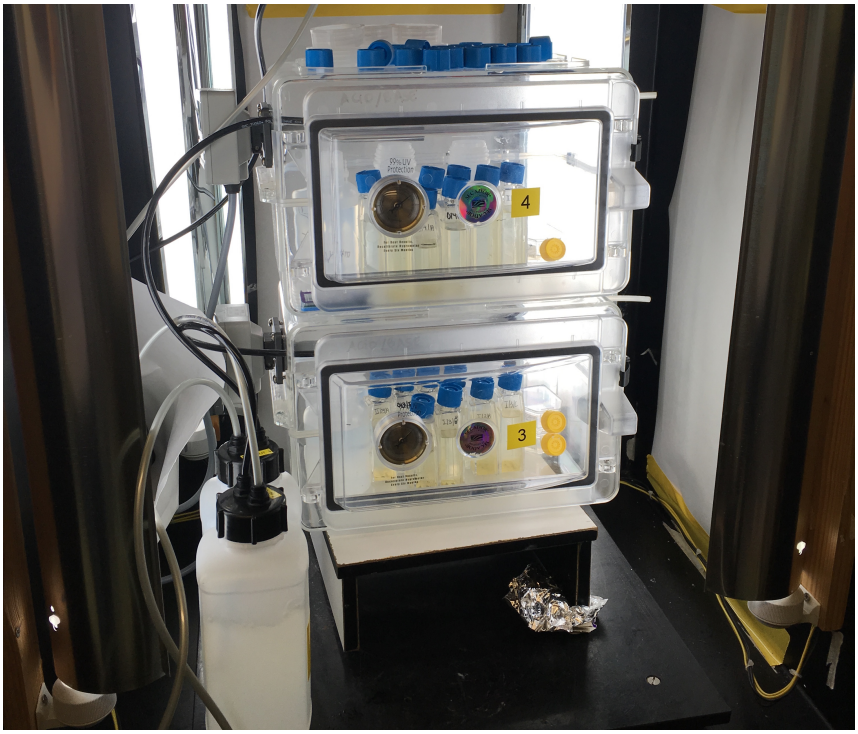

Figure 5. Photograph showing two out of the four chambers with the culture flasks hosting the foraminifera. Additionally, the four light sources and the bottles filled with MiliQ, leading to humidified air within the chambers, are shown.

The availability of living foraminifera resulted in varying starting dates of different treatments, primarily due to the high mortality rates during storage within the incubator. Furthermore, three replicates of *A. lessonii* (of 50 specimen each) per CO_2 condition and two replicates of *H.depressa* (of 25 specimen each) per CO_2 condition were set up (Fig. 3). At least one replicate per species and CO_2 condition was pre-stained with Calcein. For an overview of the experimental set-up see Table 1.

Table 1. Overview of the experiment set-up.

Chamber	Species	Number of individuals per flask	Replicate	Pre-stained with Calcein	Start date	End date
400ppm CO_2	*A. lessonii*	50	A	Yes	14.09.17	03.11.17
400ppm CO_2	*A. lessonii*	50	B	No	15.09.17	03.11.17
400ppm CO_2	*A. lessonii*	50	C	No	15.09.17	03.11.17
400ppm CO_2	*H. depressa*	25	A	Yes	21.09.17	03.11.17
400ppm CO_2	*H. depressa*	25	B	Yes	21.09.17	03.11.17
700ppm CO_2	*A. lessonii*	50	A	Yes	14.09.17	03.11.17
700ppm CO_2	*A. lessonii*	50	B	No	15.09.17	03.11.17
700ppm CO_2	*A. lessonii*	50	C	No	15.09.17	03.11.17
700ppm CO_2	*H. depressa*	25	A	Yes	21.09.17	03.11.17
700ppm CO_2	*H. depressa*	25	B	Yes	21.09.17	03.11.17
1000ppm CO_2	*A. lessonii*	50	A	Yes	14.09.17	03.11.17
1000ppm CO_2	*A. lessonii*	50	B	No	15.09.17	03.11.17
1000ppm CO_2	*A. lessonii*	50	C	No	15.09.17	03.11.17
1000ppm CO_2	*H. depressa*	25	A	Yes	28.09.17	03.11.17
1000ppm CO_2	*H. depressa*	25	B	No	28.09.17	03.11.17
2200ppm CO_2	*A. lessonii*	50	A	Yes	14.09.17	03.11.17
2200ppm CO_2	*A. lessonii*	50	B	No	15.09.17	03.11.17
2200ppm CO_2	*A. lessonii*	50	C	No	15.09.17	03.11.17
2200ppm CO_2	*H. depressa*	25	A	Yes	21.09.17	03.11.17
2200ppm CO_2	*H. depressa*	25	B	No	28.09.17	03.11.17

3. Measurements and seawater parameters

Once a week, the Falcon® bottles with the foraminifera were taken out of the chambers and water samples from every condition and replicate were taken. Afterwards, the bottles were filled up with seawater, which was incubated in the corresponding CO_2 chambers for a week in advance (initial values). Furthermore, 1mL of *Dunaliella salina* mixture (6.5mg freeze-dried *Dunaliella* on 20mL of seawater) was added to the bottles as a food source for the foraminifera. However, due to difficulties of maintaining sufficient food supply in week 42 (17.10.2017), living *Dunaliella salina* from a culture medium were fed instead of the usual mixture. The sampling time was kept as short as possible to avoid uncontrolled conditions outside of the chambers. Therefore, the Falcon® bottles were placed in the chambers again as soon as water exchange and feeding were carried out.

A part of the sampled seawater from the different conditions (CO_2, foraminifera-species and replicates) was filled up in $HgCl_2$ pre-poisoned pony-vials and stored in the fridge for later

analysis of dissolved inorganic carbon (DIC). Another part was transferred into empty pony vials and stored in the freezer for later nutrient analyses (phosphate, ammonium, nitrate, nitrogen dioxide and silicate). The remaining seawater samples were used to immediately analyze total alkalinity (TA) at the NIOZ using an automated spectrophotometric alkalinity system (ASAS) presented by Liu et al. (2015). To prevent drifts and receive a high accuracy during measurements, at each measurement day a certified reference material (Dickson) was analyzed and used for correction. For the total alkalinity measurements, at least 60mL of sample is necessary, and therefore, 20mL of seawater from each *A. lessonii* replicate was used and 30mL of seawater from each *H. depressa* replicate. Therefore, TA measurements are an average of the TA from the replicates in each chamber. Additionally, TA and nutrient sample of the water, which was incubated for a week in the corresponding chambers and used to fill up the bottles after sampling, were taken. This provides initial values without the effect of calcification.

The initial assumption was that the salinity in the bottles would not change due to the humidifying step in the experiment set-up and as vented caps were used. Nevertheless, a change in salinity was observed during the experiment. Consequently, to be able to correct for variations in salinity, salinity was determined for every sample and every week using a salinity refractometer.

The whole carbonate system was calculated from the TA and CO_2 concentrations using the software CO2SYS XLS v2.1 (Table 2) (Pierrot et al., 2006).

Calcification rates were estimated by applying the data to the alkalinity anomaly technique (Smith and Key, 1975, Langdon et al., 2010, Jacques and Pilson, 1980). Two different calculations were conducted: one without nutrients and one taking nutrients into account.

The mass of $CaCO_3$ produced (in $\boldsymbol{\mu}$g) by the two foraminiferal species was calculated with the Equations (Jacques and Pilson, 1980):

Without nutrients:

$$m(CaCO_3) = 0.5 \times \Delta A_T \times 100 \times V_{sw} \times \rho_{SW}$$

Including nutrients:

$$m(CaCO_3) = 0.5\times[\Delta A_T + \Delta PO_4 - \Delta NH_4 + \Delta(NO_3 + NO_2)]\times 100\times V_{sw}\times \rho_{SW}$$

where ΔA_T is the change in total alkalinity between the intial values without any foraminifera added to the seawater and the final values including calcifying foraminifera in seawater in $\boldsymbol{\mu}\mathrm{mol/kg}$, V_{sw} is the volume of seawater used during the titration in L (=0.06 l), ρ_{SW} is the seawater density in kg/L taking the varying salinities into account and 100 represents the molar mass of $CaCO_3$ in g/mol. If ΔA_T was negative (Week 39, pCO_2 400 ppm, *Amphistegina lessonii*; Week 42, pCO_2 2200ppm, *Heterostegina depressa*; Week 43, pCO_2 2200 ppm, *Heterostegina depressa*) it was not considered for the statistical analysis.

4. Cleaning Procedure & Chamber determination

After the culture experiment was terminated on 3rd of November 2017, all bottles were washed with MilliQ to dispatch the foraminifera and stored in the fridge. Shortly after, the remaining foraminifera in the Falcon® bottles and the reproduction events, which took place during the experiment, were counted (Table 2). Foraminiferal shells were picked out of the bottles with a brush and cleaned following the protocol based on Barker et al. (2003).

10 foraminifera were placed in 10mL PE vials and 400µL of 1% H_2O_2 solution (0.5mL H_2O_2 added to 50mL 0.1M NH_4OH) was added to each vial. Afterwards the vials were placed in a water bath at 95°C for 10 minutes to remove the organic matter. Then, the oxidizing reagent was removed. This procedure was repeated once and ultimately, the foraminiferal shells were washed five times with MilliQ. Then, they were dried under a laminar flow hood overnight.

The next day, the foraminiferal shells, which were pre-stained with Calcein (see Table 1), were analyzed under a Zeiss Axioplan 2 fluorescence microscope. The chambers built before the transfer to the experimental set-up were emitting green light, while the chambers built thereafter, during the experiment, did not emit fluorescent light (Fig. 6). Therefore, it was possible to distinguish how many chambers were newly built during the experiment in the CO_2 setup and this was recorded (Table 2).

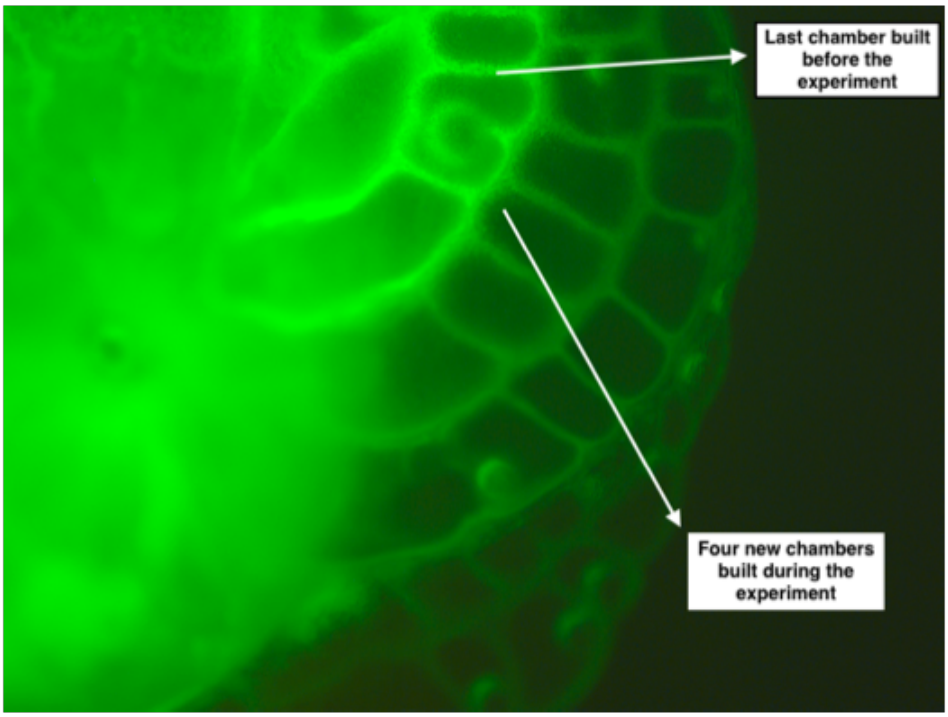

Figure 6. Heterostegina depressa. Pre-stained chambers built before the experiment are clearly fluorescent, while the four chambers built during the experiment are less/not fluorescent.

Results

All statistical analyses were conducted within the programming environment R, Version 1.0.153. To test for statistically significant differences, ANOVAs were conducted and if differences were found, a post hoc tukey test was performed to locate the differences.

1. Chambers and reproduction events

Heterostegina depressa built more chambers than *Amphistegina lessonii* (ANOVA, p=0.0195) with both species building the most chambers under 700ppm CO_2 values (see Table 2 for all CO_2 system parameters). As pre-staining did not work for the 1000ppm treatment of *H. depressa*, the data on added chambers is not available. For later comparisons and calculations, an average of the other three pCO_2 conditions of *H. depressa* was used for the 1000ppm condition (~0.47 chambers per individual per week).

The 400 and 700 ppm CO_2 treatment data of *H. depressa* is based on a larger sample size as two tissue bottles were used for the counts, while all other data of *H. depressa* and the data of *A. lessonii* is based on one tissue bottle each (Table 2). During the experiment,

reproductions took place in two treatments (400 and 700 ppm CO_2) of *A. lessonii* (Table 2). However, those reproductions are not taken into account for further analysis.

Table 2. Summary of the carbonate systems, reproduction events and chamber formations depending on pCO_2 and species. For replicates B of H. depressa no chambers per individuals per weeks are given as data from replicate A and B was combined and the combined data is shown in the row of replicate A.

Species	*A. lessonii*	*A. lessonii*	*A. lessonii*	*H. depressa*	*H. depressa*	*H. depressa*	*H. depressa*	*H. depressa*
pCO_2	400	700	1000	400	400	700	700	2200
Replicate	A	A	A	A	B	A	B	A
TA	2395.0+-9.5	2411.4+-17.9	2411.3+-14.5	2395.0+-9.5	2411.4+-17.9	2411.3+-14.5	2411.3+-14.5	2403.3+-26.5
CO_2	11.87	20.55	29.37	11.87	20.55	29.37	29.37	64.09
HCO_3^-	1856.42	2033.47	2123.64	1856.42	2033.47	2123.64	2123.64	2254.17
CO_3^{2-}	219.13	153.91	117.30	219.13	153.91	117.30	117.30	60.77
pH	8.04	7.84	7.71	8.04	7.84	7.71	7.71	7.39
$\Omega_{Calcite}$	5.18	3.62	2.76	5.18	3.62	2.76	2.76	1.43
Chambers overall	59	87	47	22	37	52	39	34
Individuals	31	30	30	9	13	15	15	12
Weeks	7	7	7	6	6	6	6	6
Reproductions	17	17	0	0	0	0	0	0
Chambers per Individual per Week	0.27	0.41	0.22	0.44*	*	*	0.51*	0.47

2. Total alkalinity and the carbon system

Prior to conducting an ANOVA, a Shapiro-Wilk normality test was conducted to test for normality (if p > 0.05 the distribution of the data is not different from a normal distribution; hence, we can assume normal distribution) within the different delta total alkalinity´s (∆TA/deltaTA) of the 4 different pCO_2 conditions and the data allowed to assume normal distribution (Shapiro-Wilk normality test: for 400ppm: p-value = 0.5992; for 700ppm: p-value = 0.8213, for 1000ppm: p-value = 0.3188, for 2200 ppm: p-value = 0.08898). The ∆TA differed significantly between the CO_2 treatments (ANOVA: p=0.0000138) and the biggest change in TA was recorded after an incubation of a week with an atmospheric CO_2 value of 700ppm (Fig. 7). A Tukey post hoc comparison revealed that only the differences between the 400&2200ppm (p=0.013), the 700&1000ppm (p=0.01) and between 700&2200ppm (p=0.000005) treatments differed significantly.

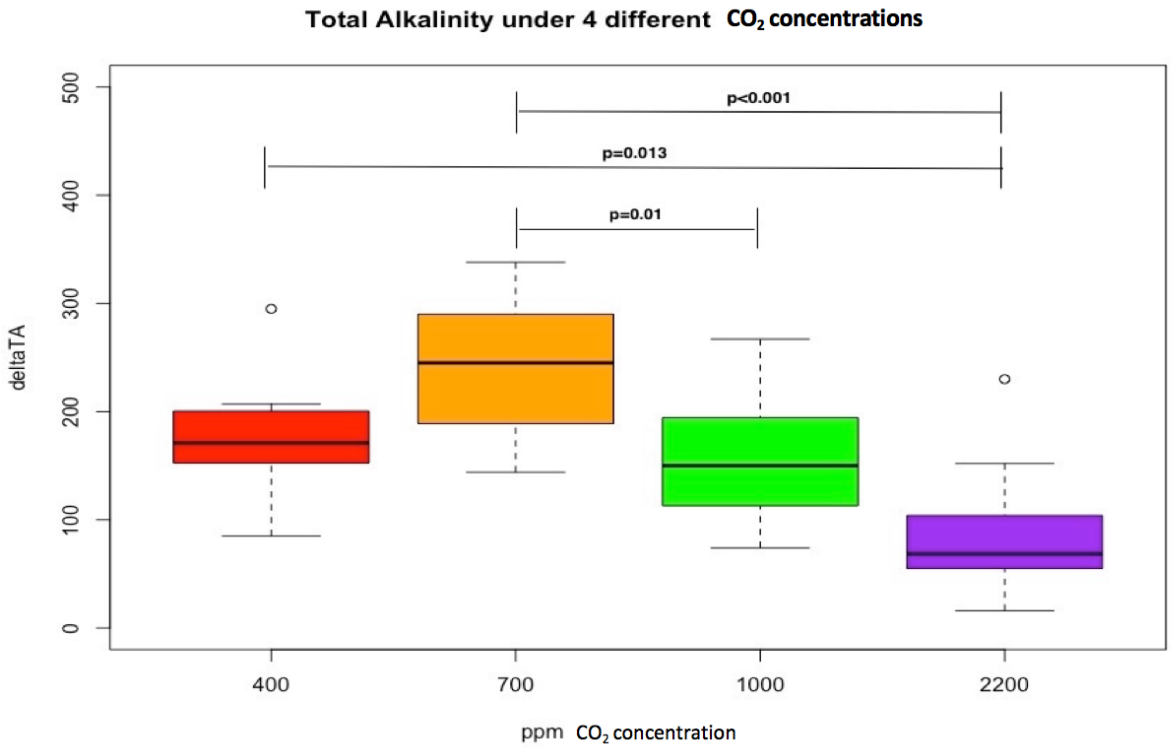

Figure 7. Delta total alkalinity (Final TA minus Initial TA) under the four different pCO_2 treatments (400ppm, 700ppm, 1000 ppm and 2200 ppm). Weeks were used as replicates. Here the results from both species were taken together. Significant differences are displayed.

The differences in ∆TA between the two foraminifera species are not significantly different (ANOVA: p=0.911) from each other. However, differences can be found between the four pCO_2 treatments within each species. For *A. lessonii* only the difference between 700&2200 ppm was statistically significant (p=0.006). For *H. depressa*, however, the change in total

alkalinity was bigger under the 700 ppm treatment than under the 2200 ppm treatment (p=0.00038). Furthermore, the alkalinity change was larger under the 1000ppm treatment than the 2200 ppm treatment (p=0.0099) and also under the 700ppm treatment compared to 400ppm treatment (p= 0.0348990) (Fig. 8). Therefore, H. depressa seems to be more successful in calcification under 700 ppm than under 400ppm and furthermore, it calcifies more under 700 and 1000 ppm atmospheric CO2 conditions than under 2200 ppm (Fig. 8).

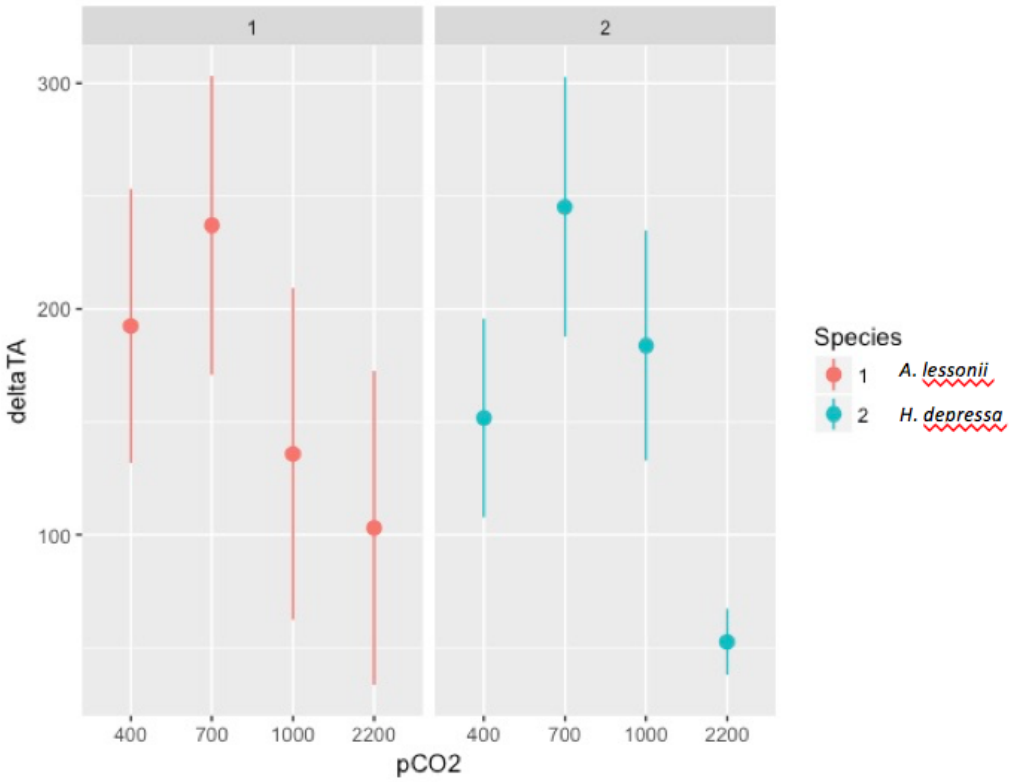

Figure 8. Change in total alkalinity between initial and final values for both species. Species 1= Amphistegina lessonii and Species 2= Heterostegina depressa. On the x-axis the four different pCO_2 treatments are plotted: 400, 700, 1000 and 2200 ppm.

Ultimately, the data was corrected for total alkalinity being influenced by nutrients. Nutrient values were in general relatively low, except for week 39, where all final (F) nutrient values were increased and furthermore, in week 40 and especially in the 1000ppm treatment, higher nutrient concentrations were detectable (Fig.13).

After correction of the total alkalinity data for nutrients and further transformation to mass $CaCO_3$ per individual, the differences between *A. lessonii* and *H. depressa* become significant (ANOVA: p=0.000375) (Fig. 9, Fig. 10). Now, no statistical significant difference can be found between the different pCO_2 treatments of *A. lessonii* (p>0.05). However, *H. depressa* produces more $CaCO_3$ in the first three lower pCO_2 treatments (400, 700 and 1000ppm) than it does at

an ambient pCO_2 of 2200 ppm (400&2200ppm: p ~ 0.012, 700 & 2200ppm: p ~ 0.002, 1000&2000ppm: p ~ 0.04) (Fig. 11). Furthermore, it is apparent that *H. depressa* produced more $CaCO_3$ than *A. lessonii* did (Fig. 11) (ANOVA: p=0.000375). The production of $CaCO_3$ of both species decreased with time in the highest pCO_2 condition (2200 ppm) (Fig. 10).

Figure 9. Nutrient samples taken every week. I= Initial values (before adding foraminifera) and F= final values (after incubation with foraminifera for a week). Furthermore, it is important to note that food was added in between I and F. Therefore, initial does not include the nutrients from the added food, while final is with food. Data for both foraminifera species are included here.

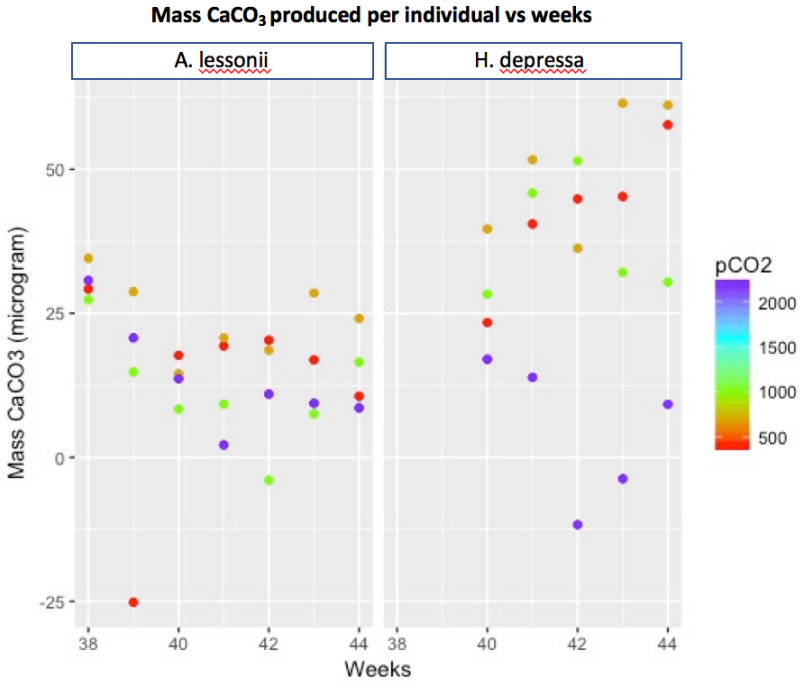

Figure 10. Mass $CaCO_3$ produced per individual including the nutrient correction. 1=Amphistegina lessonii and 2= Heterostegina depressa.

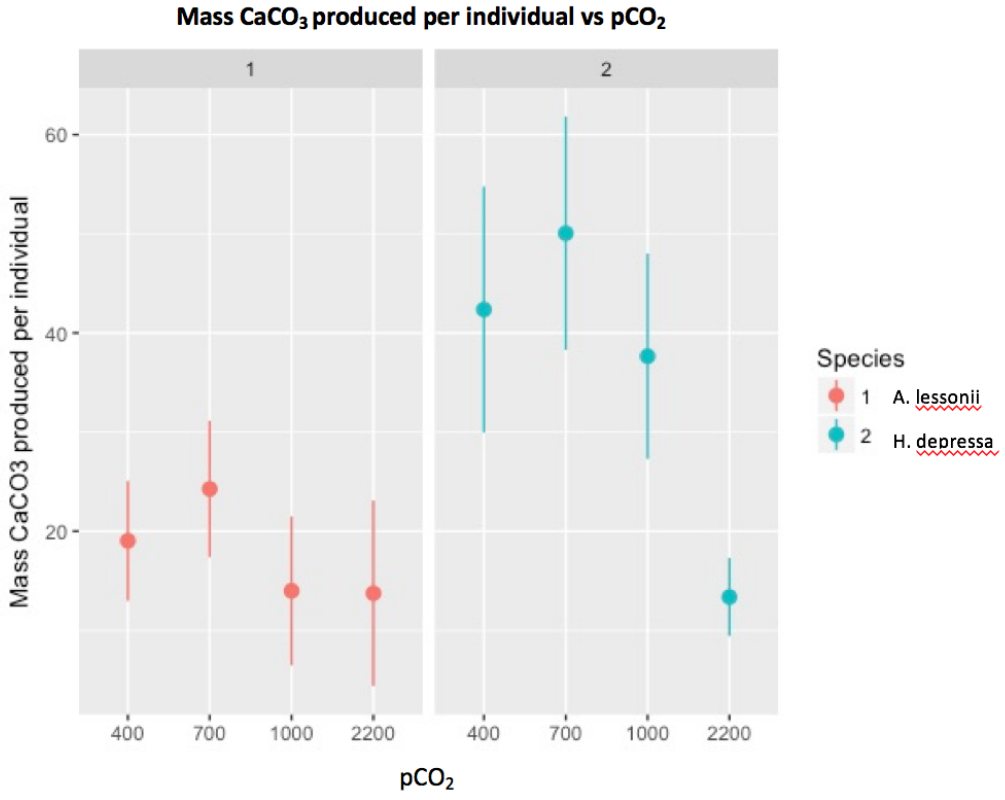

Figure 11. Mass $CaCO_3$ produced per week per individual under the four different pCO_2 treatments (400,700,1000 and 2200 ppm). The different weeks were used as replicates. This data was corrected for nutrients. Species 1= Amphistegina lessonii, Species 2= Heterostegina depressa.

This experiment shows that the two benthic photosymbiotic foraminifera *Heterostegina depressa* and *Amphistegina lessonii* are most successful in chamber formation under an atmospheric pCO_2 value of 700 ppm. Furthermore, interspecies differences can be found, as *H. depressa* built more chambers than *A. lessonii* and it produced more $CaCO_3$ (taking correction for nutrients into account) than *Amphistegina* did. A change in total alkalinity induced by biomineralization of the foraminifera suggests that it is more difficult for both species to calcify under a high pCO_2 of 2200ppm compared to 400, 700 and 1000 ppm. However, after taking the influence of nutrients into account and transforming the data into a mass of $CaCO_3$, only *H. depressa* calcifies more under the three lowest pCO_2 treatments compared to 2200ppm.

Discussion

1. Total alkalinity changes and calcification

Various experiments on foraminifera with altered seawater carbonate chemistry and ocean acidification (OA) simulations have been conducted so far and the response varied greatly (see introduction of this report). While the total alkalinity data from this experiment suggests that ambient CO_2 values up to 700ppm are beneficial for the calcification of foraminiferal shells compared to high pCO_2 values of 2200ppm, Vogel and Uthicke (2012) found that growth rates of *Amphistegina radiata* and *H. depressa* were not affected by increased pCO_2. However, their highest pCO_2 treatment was 1925 ppm, while in our experiment the highest ambient CO_2 values used were 2200ppm. Furthermore, Keul et al. (2013) point out that the methods used, as well as the species studied, can lead to varying results. The species in our experiment and the species in Vogel and Uthicke (2012) are both from the *Rotaliida* group and therefore, have low Mg-calcite tests (Gupta, 1999) and one species (*H. depressa*) used in the experiments was even identical. However, in their study, the growth rates of the specimen were determined by investigating the increase in cross-sectional surface area while we counted how many new chambers were built during the experiment and used the alkalinity anomaly technique to evaluate how much the specimen calcified. Therefore, the use of different methodologies serves as a potential explanation for the different results obtained between these two experiments. As our experiment was conducted with the same method but with two different species, it allows for comparison of species-specific effects between *A. lessonii* and *H. depressa*.

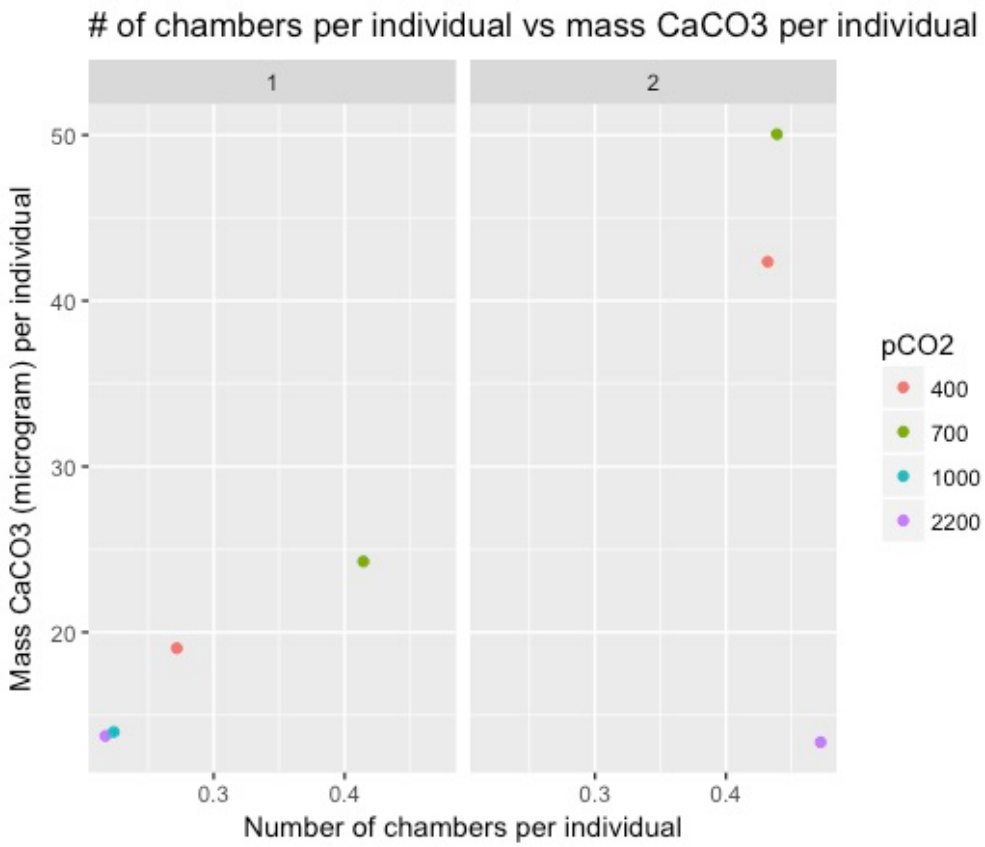

Figure 12. Average number of chambers built per individual vs mass of $CaCO_3$ produced per individual. 1= Amphistegina lessonii. 2= Heterostegina depressa. The different colours represent the four different pCO2 conditions.

The method used here is the total alkalinity anomaly technique first described by Smith and Key (1975). This method relies on the principle that for every production of a mole of $CaCO_3$, the total alkalinity (TA) decreases by two moles and the TA is not directly affected by photosynthesis or respiration (Chisholm and Gattuso, 1991, Smith and Key, 1975, Zeebe and Wolf-Gladrow, 2001). Therefore, the measurements prescribed in this report serve as a direct way of chemically determining the amount of calcification that took place during the incubations. However, two potential sources of errors have to be taken into account: Nutrients having an influence on the TA, and analytical precision. As nutrients can be used by the foraminifera and chemically altered to products which are equivalent to acid, this alters the TA (Jacques and Pilson, 1980). However, as we took nutrient samples, we were able to correct for this when we calculated the mass of $CaCO_3$ produced. The second source of error derives from the necessity of a certain amount of change in TA, to receive an accurate measurement of the mass $CaCO_3$ being produced (Langdon et al., 2010). An estimated number

of necessary foraminifera for a large enough change was calculated prior to our experiment. However, as the availability of foraminifera was a limiting factor, this implied that the volume of seawater used for the measurements with the spectrophotometer had to be kept minimal (60mL). A smaller volume of seawater leads to less accurate measurements with the machine and hence, this could have increased the error in the results. Furthermore, the number of available foraminifera did not allow for replicates of the measurements within each week but only to use the weeks as replicates. As the weekly data is based on only one replicate, the conclusions regarding weekly changes are rather inaccurate. This could also be a potential source for loosing statistical significance. The differences between the weeks, visible in Fig. 10, show that the signal is changing over the period of incubation in the experiment. Therefore, this is a potential source of an increased variability of our data and loss of statistical significance. Further studies are needed to accurately investigate the influence of the time of exposure to different pCO_2 conditions. Nevertheless, using the weeks as replicates and correcting for nutrient concentrations, increases the accuracy of our results and allows for valid interpretations of the effect of pCO_2 on calcification of two foraminiferal species.

The total alkalinity data suggests that *A. lessonii* calcifies more under 700 ppm, than under 2200 ppm and that *H. depressa* calcifies more under 700 ppm than under 400 ppm, but also that the alkalinity change is greater under the 700 and 1000 ppm treatment compared to 2200 ppm. Overall, although not always statistically significant, the trend suggests that the ideal pCO_2 concentration for calcification for the two foraminiferal species used in this study is around 700 ppm and that calcification is impaired under very high pCO_2 concentrations of 2200 ppm (Fig. 8). However, here the number of individuals who calcified during the experiment and the correction for nutrients is not taken into consideration yet. After converting the data to a mass of $CaCO_3$ produced per individual and correcting for nutrients, a statistically significant difference in calcification between the two species can be found. This could be due to size differences between the two foraminifera, with *H. depressa* being larger than *A. lessonii.* Apart from the difference between the two species, the interpretation of the effect of pCO_2 on the calcification of each species has to be revised as well. Although, for *A. lessonii* the 700ppm CO_2 treatment still seems to be beneficial for calcification (Fig. 11), especially compared to the higher pCO_2 treatments, this trend is not statistically significant anymore. However, comparing the mass of $CaCO_3$ produced to the number of chambers built per individual, the suggestion of an enhanced calcification under 700 ppm CO_2 conditions in

this species is supported (Fig. 12). For the second species, *H. depressa*, the converted and corrected data show a clear and significant decrease in mass of $CaCO_3$ produced per individual in the highest CO_2 treatment of 2200ppm, compared to the three lower CO_2 conditions (Fig. 11). Here, the chamber counts agree with the TA data for the 400 and the 700 ppm treatment (no data on chambers formed under 1000 ppm available due to lack of pre-stained individuals), but the chamber count of *H. depressa* in the 2200 ppm treatment is much higher than TA data suggests (Fig. 12). A possible explanation could be that the chambers of those individuals were built during the first two weeks in the experiment, when *H. depressa* still seemed to be able to cope better with the high pCO_2 conditions (Fig. 10). Furthermore, only one replicate/bottle was pre-stained, but the TA data was derived to equal parts from both replicates. Therefore, if the specimen in the second replicate calcified less than in the first, this could also explain decreased change in TA compared to the chamber counts. Furthermore, the specimen could have built a high number of chambers, but they could be thinner. The differences found between the two species also suggest, that *A. lessonii* is able to cope better with high pCO_2 values of 2200ppm than *H. depressa*.

Fujita et al. (2011) also investigated the effect of pCO_2 on two hyaline foraminiferal species with diatom symbionts. However, they did not use *A. lessonii* and *H. depressa*, but *Baculogypsina sphaerulata* and *Calcarina gaudichaudii*, two high Mg-Calcite species. Their results suggest a similar trend as ours, as net calcification of their foraminifera increased under a pCO_2 of 580 and 770 ppm compared to 260 ppm, but decreased again under the highest pCO_2 used in their experiment of 970 ppm (Fujita et al., 2011). Therefore, their and our study taken together, both suggest that calcification in hyaline foraminiferal species is still equally successful or even enhanced under higher pCO_2 conditions than present, but is impaired under a very high CO_2 world (of 970ppm for *B. sphaerulata* and *C. gaudichaudii* and 2200ppm for *A. lessonii* and *H. depressa*). Fujita et al. (2011) suggest that the successful calcification, even under higher pCO_2 levels, might be coupled to a higher DIC availability. However, while under those conditions DIC availability increases, the pH reduces, and therefore, carbonate ion availability decreases (Ridgwell and Zeebe, 2005, Zeebe and Wolf-Gladrow, 2001). If the precipitation of calcium carbonate in foraminifera would mainly depend on the concentration of calcium and carbonate, this would imply a hampered calcite precipitation under higher DIC concentrations (Zeebe and Wolf-Gladrow, 2001). Our experiment therefore suggests that foraminifera can also use other DIC sources apart from carbonate during calcification.

2. Biomineralization

As earlier experiments on foraminifera have shown that the $CaCO_3$ formed by foraminifera varies significantly from carbonate formed by inorganic precipitation (e.g.: Lowenstam and Weiner, 1989), various biomineralization models for foraminiferal calcification have been established so far. These are based on the principle of "the vital effect" (Urey et al., 1951, Weiner and Dove, 2003): the biological control leading to a disequilibrium between inorganic precipitation and elemental incorporation in the foraminiferal tests. To make $CaCO_3$ tests foraminifera need to elevate the carbonate and calcium concentrations at the site of calcification and simultaneously decrease the concentration of crystallization inhibitors such as magnesium (de Nooijer et al., 2014, de Nooijer et al., 2009). Therefore, the proposed mechanism has to combine the ability to elevate the carbonate concentration relative to the magnesium concentration (de Nooijer et al., 2009), but at the same time it must be possible to calcify under higher pCO_2 concentrations and therefore, lower carbonate concentrations (as suggested in our experiment). Additionally, it must be considered, that as $CaCO_3$ is being precipitated, the calcifying fluid acidifies and therefore, without any regulation, it would lead to a negative feedback for calcification (Glas et al., 2012, Zeebe and Wolf-Gladrow, 2001).

Seawater vacuolization and transmembrane ion transport are the main two accepted biomineralization models nowadays (Evans et al., 2018, de Nooijer et al., 2014). While the first assumes that the necessary ions are derived from vacuolizing the seawater (e.g.: Bentov et al., 2009, Ter Kuile and Erez, 1988, Evans et al., 2018), the latter hypothesizes active transport of calcium through channels or pumps to the site of calcification (Toyofuku et al., 2017, Glas et al., 2012, de Nooijer et al., 2009).

Recently, for the vacuolization model an active pH regulation of the vacuolized seawater was suggested (Bentov et al., 2009): After the seawater is vacuolized, protons are being transported out of it and into the cytosol. As the pH is higher in the vacuole, CO_2 from the cytosol diffuses in and transforms to carbonate. It is difficult to explain with this model why magnesium concentrations of foraminiferal calcite are lower than they would be if they followed inorganic precipitation, but different hypotheses have been established (e.g.: Erez, 2003). Active pH regulation can, however, explain the concentration of carbonate at the site of calcification and was already suggested for various species of foraminifera (de Nooijer et al., 2009, Glas et al., 2012).

Toyofuku et al. (2017) were able to explain the mechanism of calcification in *Ammonia sp.*: they found a V-type H^+ ATP-ase as a transmembrane pump leading to an outward proton flux. This outward proton flux results in a lowered pH in the microenvironment outside of the foraminiferal test and therefore, the carbonate chemistry changes to more CO_2 availability. With higher availability of carbon-dioxide the diffusion gradient to the site of calcification increases. Inside the site of calcification, the pH is higher due to the outward proton pumping and therefore, the diffused CO_2 is being transformed to the CO_3^{2-} necessary for $CaCO_3$ precipitation (Toyofuku et al., 2017).

To explain how *A. lessonii* and *H. depressa* can calcify equally good or even better at higher pCO_2 concentrations than modern, some kind of active pH regulation is necessary. Therefore, models without active pH regulation can be excluded for those two species. Whether this alkalization happens within vacuoles, or by active proton pumping over the protective envelope or a combination of both, cannot be derived from this study. However, Bentov et al. (2009) suggest for the vacuolization model with active pH regulation that foraminiferal calcification would be reduced at higher atmospheric CO_2 concentrations, because more energy is needed to reduce the pH and transform CO_2 to carbonate in more acidic vacuolized seawater. Therefore, the trans membrane transport of calcium and the active proton pumping over the whole surface of the protective envelope are the preferred hypotheses here.

Under an "intermediate" pCO_2 value of 700ppm (and 1000 ppm for *H. depressa*), the foraminifera prosper as there is a higher CO_2 availability in the seawater thus, more of which diffuses to the calcification site. As the carbon-dioxide is being transformed to carbonate inside the foraminifera, due to a higher intracellular pH, it results in enhanced $CaCO_3$ precipitation ("mass $CaCO_3$ produced" in our experiment). However, I propose for this experiment, that under an even higher ambient pCO_2 of 2200 ppm, the seawater-pH external to the foraminiferal shell is already lowered and achieving an internal pH by outward proton pumping that is high enough for carbon dioxide to be transformed to carbonate at the site of calcification demands too much energy to still be efficient. Therefore, the energy costs of proton pumping outweigh the energy gains by an increased DIC availability and calcification is hampered. This particularly seems to be the case for *H. depressa*. As the differences between the different pCO_2 treatments were not significant for *A. lessonii*, it might indicate that the threshold between energy gain and costs was not surpassed during our experiment. However,

the chamber counts suggest that also for this species calcification was more successful especially under 700 ppm than under 1000 and 2200 ppm (Fig. 12).

Some differences between the two foraminiferal species used in this experiment could also be attributed to the different sizes of the two species. With *H. depressa* having a larger surface test area but being much "flatter", the surface to volume ratio of this species is higher compared to *A. lessonii*. This results in more diffusion of CO_2 to the site of calcification and therefore, more $CaCO_3$ can be produced in the same amount of time. Also, each chamber of *H. depressa* is bigger than the chambers of *A. lessonii*, implying that more $CaCO_3$ was precipitated during a chamber formation event. This could particularly explain why more mass of $CaCO_3$ was produced in the bigger species (Fig. 11), while the number of chambers formed was similar between both species (Fig. 12).

Table 3. The amount of protons pumped for one single chamber in our experiment. Here the comparison between the 400ppm and the 2200ppm condition is shown for both species.

pCO_2	**Species**	**$CaCO_3$ [gram]**	**$CaCO_3$ [micromoles]**	**Protons Pumped [micromoles]**
400	*A. lessonii*	69.999	0.699	1.399
2200	*A. lessonii*	54.191	0.5414	1.083
400	*H. depressa*	114.441	1.143	2.287
2200	*H. depressa*	28.305	0.283	0.566

As we explain our results with the mechanism suggested by Toyofuku et al. (2017), a comparison of our data with their data on *Ammonia sp.* was attempted. For this purpose, the $CaCO_3$ produced during the formation of one chamber was calculated (Table 3) for the lowest (400ppm) and highest (2200ppm) treatments of our experiment. For both species, it was found that under the lower pCO_2 treatment of 400 ppm more protons were pumped than under the highest treatment (Table 3). Furthermore, *H. depressa* pumped more protons under 400 ppm than *A. lessonii* did, but *H. depressa* was less successful in proton pumping under the highest pCO_2 concentration.

The data was transformed to the amount of protons pumped from the site of calcification to the seawater outside of the protective envelope assuming for one mole of $CaCO_3$ produced two moles of protons were pumped out. The resulting amount of protons are displayed in Table 3 in micrograms. Toyofuku et al. (2017) found a cumulative proton flux of approx. 55 picomoles per chamber formed (see Fig. 2 in Toyofuku et al., 2017). Therefore, our numbers

are about two orders of magnitude bigger than the numbers from Toyofuku et al. (2017). However, two important factors have to be taken into account for this comparison:

1. Some of the protons pumped out are immediately being transformed to H_2CO_3 as they react with the bicarbonate in the water and some immediately diffuse. Toyofuku et al. (2017) were able to account for those two processes using diffusion equations, while we were not able to account for them due to a lack of data on the distribution of protons in those two species during chamber formation.
2. *Ammonia sp.* is a smaller species than *A. lessonii* and *H. depressa* and while Toyofuku et al. (2017) did not notice a "clear relation between the foraminiferal size and the pH reduction" they state that "specimens with the largest diameter were associated with the highest total proton flux." (Toyofuku et al., 2017, p.3). As the diameter of the species used in our experiment is larger, a higher total proton flux can therefore be expected.

Another important aspect about the two foraminiferal species used in this study is to consider that both of them have photosymbionts. The photosynthesis rate of these photosymbionts might increase under elevated DIC concentrations (Fujita et al., 2011), as CO_2 is being used up to produce glucose (and oxygen). Additionally, the photosymbionts release CO_2 at night. Fujita et al. (2011) furthermore suggest that the produced glucose is an energy source for the foraminiferal host (Hallock, 2000) and might be used for the primary organic sheet (Gupta, 1999). Bé et al. (1982) even found that an elimination of symbionts in *G. sacculifer* led to reduced chamber formation rates or shorter survival times. The results of our experiment compared to experiments conducted on non-photosymbiont bearing foraminifera also suggest that photosymbionts could have a major effect on chamber formation. Dissard et al. (2010) and Keul et al. (2013) both found that the shell weights of the symbiont-free foraminifera *Ammonia sp.* decreased with decreasing carbonate concentrations. This may indicate that the increase or consistency of calcification in our experiment at a higher pCO_2 compared to current values and hence, lower carbonate concentrations, could also be partly attributed to the photosynthetic activity and enhancement of foraminiferal calcification by photosymbionts.

In corals, a very similar calcification model to the one used in Toyofuku et al. (2017) is suggested (Sinclair and Risk, 2006, McConnaughey and Whelan, 1997): CO_2 diffuses into the calcifying fluid from the coelenteron and an ATPase is pumping Calcium ions into the calcifying

fluid and protons out of it to increase the pH (see Fig. 1B in McConnaughey and Whelan (1997)). Due to the resulting alkaline conditions at the site of calcification, the available CO_2 is being transformed to carbonate, which is available for $CaCO_3$ formation. As this leads to proton formation and subsequently pumping into the coelentric fluid, the protons react with the bicarbonate and produce more CO_2. This CO_2 in turn is believed to be used by the symbiotic zooxanthella for photosynthesis (McConnaughey and Whelan, 1997, Sinclair and Risk, 2006).

3. The past and the future

Investigating the evolution of a species can give further insights about its ecological niche and its adaptations to the environment. The ecological niche was first described by Hutchinson (1991) as the "hypervolume" in which abiotic and biotic environmental parameters allow a species´ population to persist (Holt, 2009). According to niche conservatism, species tend to keep their ecological niches over time (Wiens et al., 2010). Therefore, the conditions in which an organism used to live in the past can be used to improve our knowledge and understanding of its modern capabilities.

A. lessonii and *H. depressa* are both low Mg-calcite *Rotaliida* foraminifera (Gupta, 1999). The order of *Rotaliida* appeared for the first time in the Cambrian (Pawlowski et al., 2003). Berner and Kothavala (2001) modelled Paleozoic levels of CO_2 and suggest that pCO_2 during the Cambrian was approximately 25 times higher than today (taking a value of 300ppm as "today") (see Fig. 13 in Berner and Kothavala (2001)). Hence, if calcifying foraminifera formed under atmospheric conditions with such high CO_2 values they had to have adaptive mechanisms to high pCO_2 values. Further evidence of the capability to survive under high CO_2 concentrations can be found in Van Dijk et al. (2016). They investigated the abundances of foraminifera with varying mineralogy, namely high magnesium calcite, low magnesium calcite and aragonite, during the Phanerozoic and showed a correlation with Mg/Ca concentrations of seawater (Van Dijk et al., 2016). The abundance of low magnesium calcite species, as *A. lessonii* and *H. depressa* are, coincides with low Mg/Ca concentrations in the ocean which were present from the late Cambrian until the early Carboniferous and further from the mid-Jurassic until the mid-Cretaceous (Van Dijk et al., 2016).

Apart from the differences in Mg/Ca content of the seawater, those periods also coincide with major changes in CO_2 concentrations according to climate reconstructions. From the late Cambrian to the early Carboniferous, CO_2 concentrations were much higher than today and

from the mid-Jurassic to the mid-Cretaceous they were slightly higher, while in periods with dominance of high magnesium calcite or aragonite forming foraminifera, CO_2 concentrations tended to be lower (Berner and Kothavala, 2001). Therefore, to our knowledge, benthic low magnesium calcite foraminifera seem to be able to survive, and even thrive, under high CO_2 concentrations and low Magnesium/Calcium concentrations of seawater. Their evolution and paleo-distribution suggests mechanisms which enable them to grow under higher CO_2 concentrations than modern.

As the chemical and isotopic composition of foraminiferal tests is used in paleoclimatic studies to reconstruct past oceanic conditions, it is necessary to understand the vital effects influencing and changing the chemistry recorded in the shells. Even though both species used in our experiment are benthic, hyaline, photosymbiont bearing foraminifera from the *Rotadliid* group, species-specific differences were found. This suggests that even though they might use the same mechanism for biomineralization, the threshold between energy gain and loss through increased DIC availability could still be varying. To understand this better, an investigation of the pH under different ambient pCO_2 conditions with different foraminifera during chamber formation (for example with microsensor studies) is needed.

Furthermore, the results from this study have important implications for the future of those marine calcifiers under a more acidic ocean. While initially often assumed that higher DIC and lower pH would hamper calcification in foraminifera, with the rising number of evidence for a proton expulsion mechanism in many foraminiferal species, the presumed effects of a more acidic ocean are changing. An increased CO_2 concentration might actually enhance calcification by enhancing CO_2 diffusion rates and thereby, increasing the bicarbonate availability at the site of calcification (Toyofuku et al., 2017). However, as de Nooijer et al. (2009) point out, the decrease of the seawater pH could also counteract this effect as it becomes more difficult to pump protons under a bigger pH gradient.

Conclusion

For the first time, total alkalinity was directly measured and used to derive the calcification rates of two foraminiferal species for a present and future, more acidic ocean. Importantly, the pH of the seawater was not changed by titrations, but instead the atmospheric CO_2 concentrations were adjusted, simulating a more realistic approach.

Our results show that *A. lessonii* and *H. depressa* will be able to maintain their calcification rates at least until an atmospheric pCO_2 concentration of 700 ppm, which, in the worst IPCC scenario, will already be reached around the year 2080. However, very high levels of 2200 ppm pCO_2 would lead to impaired biomineralization, especially in *H. depressa*.

As ocean acidification does not immediately and directly lead to decreased calcification rates, a biomineralization model including active pH regulation is necessary to explain our results. Whether this process is taking place via direct trans-membrane ion uptake, via vacuolization or a combination of both, was not subject of this experiment and can therefore, not be derived from it. However, biomineralization models without any active pH regulation by the foraminifera cannot account for our results.

Acknowledgments

First and foremost, I would like to thank my parents for their emotional and financial support throughout my years of studying –*Hvala, mama i tata*. Secondly, many thanks to my partner Michael Latschbacher for always pushing and supporting me in every aspect.

I would also like to thank my supervisors Dr. Gert-Jan Reichart, Dr. Lennart de Nooijer and Linda Dämmer who made this project possible and supported me with helpful comments on my draft. Further, many thanks to Alice Webb and Inge van Dijk who helped me in various aspects from research planning to implementation and interpretation and made my time at the NIOZ and in Curacao entertaining. Great thanks to the fantastic staff at the NIOZ- Bob Koster, Sharyn Ossebaar, Karel Bakker, Wim Boer, Eric Epping, Kirsten Kooijman, Henk de Stitger and Esmee Geerken- for support in various aspects. Furthermore, I would like to thank the Arnhem Zoo for providing my foraminifera and the CARMABI Foundation for allowance to use their facilities. Last but not least, a big thank you to all my friends who were always supportive, read my drafts (Robyn), made my time in Curacao unforgettable (Chris, Nina, Fabien & Martyna) and helped me solving mathematical equations- which were not used in the end (Becko).

References

Adl, S. M., Simpson, A. G. B., Farmer, M. A., Andersen, R. A., Anderson, O. R., Barta, J. R., Bowser, S. S., Brugerolle, G., Fensome, R. A., Fredericq, S., James, T. Y., Karpov, S., Kugrens, P., Krug, J., Lane, C. E., Lewis, L. A., Lodge, J., Lynn, D. H., Mann, D. G., McCourt, R. M., Mendoza, L., Moestrup, Ø., Mozley-Standridge, S. E., Nerad, T. A., Shearer, C. A., Smirnov, A. V., Spiegel, F. W. & Taylor, M. F. J. R. 2005. The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. *Journal of Eukaryotic Microbiology,* 52**,** 399-451.

Barker, S., Greaves, M. & Elderfield, H. 2003. A study of cleaning procedures used for foraminiferal Mg/Ca paleothermometry. *Geochemistry, Geophysics, Geosystems,* 4.

Bé, A. W. H., Spero, H. J. & Anderson, O. R. 1982. Effects of symbiont elimination and reinfection on the life processes of the planktonic foraminifer Globigerinoides sacculifer. *Marine Biology,* 70**,** 73-86.

Bentov, S., Brownlee, C. & Erez, J. 2009. The role of seawater endocytosis in the biomineralization process in calcareous foraminifera. *Proceedings of the National Academy of Sciences,* 106**,** 21500-21504.

Berner, R. A. & Kothavala, Z. 2001. Geocarb III: A revised model of atmospheric CO2 over phanerozoic time. *American Journal of Science,* 301**,** 182-204.

Bernhard, J. M., Blanks, J. K., Hintz, C. J. & Chandler, G. T. 2004. Use of the fluorescent calcite marker calcein to label foraminiferal tests. *Journal of foraminiferal Research,* 34**,** 96-101.

Chisholm, J. R. & Gattuso, J. P. 1991. Validation of the alkalinity anomaly technique for investigating calcification of photosynthesis in coral reef communities. *Limnology and Oceanography,* 36**,** 1232-1239.

de Nooijer, L. J., Spero, H. J., Erez, J., Bijma, J. & Reichart, G. J. 2014. Biomineralization in perforate foraminifera. *Earth-Science Reviews,* 135**,** 48-58.

de Nooijer, L. J., Toyofuku, T. & Kitazato, H. 2009. Foraminifera promote calcification by elevating their intracellular pH. *Proceedings of the National Academy of Sciences of the United States of America,* 106**,** 15374-15378.

Dissard, D., Nehrke, G., Reichart, G. J. & Bijma, J. 2010. Impact of seawater pCO2 on calcification and Mg/Ca and Sr/Ca ratios in benthic foraminifera calcite: results from culturing experiments with Ammonia tepida. *Biogeosciences,* 7**,** 81-93.

Doney, S. C., Fabry, V. J., Feely, R. A. & Kleypas, J. A. 2009. Ocean acidification: the other CO2 problem. *Annual Review of Marine Science,* 1**,** 169-92.

Erez, J. 2003. The Source of Ions for Biomineralization in Foraminifera and Their Implications for Paleoceanographic Proxies. *Reviews in Mineralogy and Geochemistry,* 54**,** 115-149.

Ernst, S., Janse, M., Renema, W., Kouwenhoven, T., Goudeau, M.-L. & Reichart, G.-J. 2011. Benthic foraminifera in a large Indo-Pacific coral reef aquarium. *Journal of Foraminiferal Research,* 41**,** 101-113.

Evans, D., Müller, W. & Erez, J. 2018. Assessing foraminifera biomineralisation models through trace element data of cultures under variable seawater chemistry. *Geochimica et Cosmochimica Acta*.

Fujita, K., Hikami, M., Suzuki, A., Kuroyanagi, A., Sakai, K., Kawahata, H. & Nojiri, Y. 2011. Effects of ocean acidification on calcification of symbiont-bearing reef foraminifers. *Biogeosciences,* 8**,** 2089-2098.

Glas, M. S., Langer, G. & Keul, N. 2012. Calcification acidifies the microenvironment of a benthic foraminifer (Ammonia sp.). *Journal of Experimental Marine Biology and Ecology,* 424**,** 53-58.

Gupta, B. K. S. 1999. *Modern foraminifera*, Springer.

Hallock, P. 2000. Symbiont-Bearing Foraminifera: Harbingers of Global Change? *Micropaleontology,* 46**,** 95-104.

Heine, J. N. 1986. *Blue water diving guidelines,* University of California, La Jolla.

Hoegh-Guldberg, O., Mumby, P. J., Hooten, A. J., Steneck, R. S., Greenfield, P., Gomez, E., Harvell, C. D., Sale, P. F., Edwards, A. J., Caldeira, K., Knowlton, N., Eakin, C. M., Iglesias-Prieto, R., Muthiga, N., Bradbury, R. H., Dubi, A. & Hatziolos, M. E. 2007. Coral Reefs Under Rapid Climate Change and Ocean Acidification. *Science,* 318**,** 1737-1742.

Holt, R. D. 2009. Bringing the Hutchinsonian niche into the 21st century: Ecological and evolutionary perspectives. *Proceedings of the National Academy of Sciences,* 106**,** 19659-19665.

Hutchinson, G. E. 1991. Population studies: Animal ecology and demography. *Bulletin of Mathematical Biology,* 53**,** 193-213.

IPCC 2013. Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. *In:* STOCKER, T. F., D. QIN, G.-K. PLATTNER, M. TIGNOR, S.K. ALLEN, J. BOSCHUNG, A. NAUELS, Y. XIA, V. BEX AND P.M. MIDGLEY (ed.). Cambridge, United Kingdom and New York, NY, USA.

Jacques, T. G. & Pilson, M. E. Q. 1980. Experimental ecology of the temperate scleractinian coral Astrangia danae I. Partition of respiration, photosynthesis and calcification between host and symbionts. *Marine Biology,* 60**,** 167-178.

Keul, N., Langer, G., de Nooijer, L. J. & Bijma, J. 2013. Effect of ocean acidification on the benthic foraminifera Ammonia sp is caused by a decrease in carbonate ion concentration. *Biogeosciences,* 10**,** 6185-6198.

Köhler-Rink, S. & Kühl, M. 2005. The chemical microenvironment of the symbiotic planktonic foraminifer Orbulina universa. *Marine Biology Research,* 1**,** 68-78.

Kuroyanagi, A., Kawahata, H., Suzuki, A., Fujita, K. & Irie, T. 2009. Impacts of ocean acidification on large benthic foraminifers: Results from laboratory experiments. *Marine Micropaleontology,* 73**,** 190-195.

Langdon, C., Gattuso, J.-P. & Andersson, A. J. 2010. Measurements of calcification and dissolution of benthic organisms and communities. *In:* RIEBESELL, U., FABRY, V. J., HANSSON, L. & GATTUSO, J.-P. (eds.) *Guide to best practices for ocean acidification research and data reporting.* Luxembourg: Publications Office of the European Union.

Liu, X., Byrne, R. H., Lindemuth, M., Easley, R. & Mathis, J. T. 2015. An automated procedure for laboratory and shipboard spectrophotometric measurements of seawater alkalinity: Continuously monitored single-step acid additions. *Marine Chemistry,* 174**,** 141-146.

Lowenstam, H. & Weiner, S. 1989. *On biomineralization*, Oxford University Press, New York.

McConnaughey, T. & Whelan, J. 1997. Calcification generates protons for nutrient and bicarbonate uptake. *Earth-Science Reviews,* 42**,** 95-117.

Mucci, A. 1983. The solubility of calcite and aragonite in seawater at various salinities, temperatures, and one atmosphere total pressure. *American Journal of Science,* 283**,** 780-799.

Ogawa, T. & Kaplan, A. 1987. The stoichiometry between CO2 and H+ fluxes involved in the transport of inorganic carbon in cyanobacteria. *Plant physiology,* 83**,** 888-891.

Parfrey, L. W., Lahr, D. J. G., Knoll, A. H. & Katz, L. A. 2011. Estimating the timing of early eukaryotic diversification with multigene molecular clocks. *Proceedings of the National Academy of Sciences of the United States of America,* 108**,** 13624-13629.

Pawlowski, J., Holzmann, M., Berney, C., Fahrni, J., Gooday, A. J., Cedhagen, T., Habura, A. & Bowser, S. S. 2003. The evolution of early Foraminifera. *Proceedings of the National Academy of Sciences,* 100**,** 11494-11498.

Pierrot, D., Lewis, E. & Wallace, D. W. R. 2006. MS Excel Program Developed for CO2 System Calculations. ORNL/CDIAC-105a. Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, U.S. Department of Energy, Oak Ridge, Tennessee.

Ridgwell, A. & Zeebe, R. E. 2005. The role of the global carbonate cycle in the regulation and evolution of the Earth system. *Earth and Planetary Science Letters,* 234**,** 299-315.

Rink, S., Kühl, M., Bijma, J. & Spero, H. J. 1998. Microsensor studies of photosynthesis and respiration in the symbiotic foraminifer Orbulina universa. *Marine Biology,* 131**,** 583-595.

Schiebel, R. 2002. Planktic foraminiferal sedimentation and the marine calcite budget. *Global Biogeochemical Cycles,* 16**,** 3-1-3-21.

Sinclair, D. J. & Risk, M. J. 2006. A numerical model of trace-element coprecipitation in a physicochemical calcification system: Application to coral biomineralization and trace-element 'vital effects'. *Geochimica et Cosmochimica Acta,* 70**,** 3855-3868.

Smith, S. V. & Key, G. S. 1975. Carbon dioxide and metabolism in marine environments. *Limnology and Oceanography,* 20**,** 493-495.

Ter Kuile, B. & Erez, J. 1988. The size and function of the internal inorganic carbon pool of the foraminifer Amphistegina lobifera. *Marine Biology,* 99**,** 481-487.

Toyofuku, T., Matsuo, M. Y., de Nooijer, L. J., Nagai, Y., Kawada, S., Fujita, K., Reichart, G.-J., Nomaki, H., Tsuchiya, M., Sakaguchi, H. & Kitazato, H. 2017. Proton pumping accompanies calcification in foraminifera. *Nature Communications,* 8**,** 14145.

Urey, H. C., Lowenstam, H. A., Epstein, S. & McKinney, C. R. 1951. Measurement of paleotemperatures and temperatures of the upper cretaceous of England, Denkmark and the southeastern United States. *GSA Bulletin,* 62**,** 399-416.

Van Dijk, I., De Nooijer, L. J., Hart, M. B. & Reichart, G. J. 2016. The long-term impact of magnesium in seawater on foraminiferal mineralogy: Mechanism and consequences. *Global Biogeochemical Cycles,* 30**,** 438-446.

Vogel, N. & Uthicke, S. 2012. Calcification and photobiology in symbiont-bearing benthic foraminifera and responses to a high CO2 environment. *Journal of Experimental Marine Biology and Ecology,* 424-425**,** 15-24.

Weiner, S. & Dove, P. M. 2003. An Overview of Biomineralization Processes and the Problem of the Vital Effect. *Reviews in Mineralogy and Geochemistry,* 54**,** 1-29.

Wiens, J. J., Ackerly, D. D., Allen, A. P., Anacker, B. L., Buckley, L. B., Cornell, H. V., Damschen, E. I., Jonathan, D. T., Grytnes, J. A., Harrison, S. P., Hawkins, B. A., Holt, R. D., McCain, C. M. & Stephens, P. R. 2010. Niche conservatism as an emerging principle in ecology and conservation biology. *Ecology Letters,* 13**,** 1310-1324.

Zeebe, R. E., Bijma, J., Hönisch, B., Sanyal, A., Spero, H. J. & Wolf-Gladrow, D. A. 2008. Vital effects and beyond: a modelling perspective on developing palaeoceanographical proxy relationships in foraminifera. *Geological Society, London, Special Publications,* 303**,** 45-58.

Zeebe, R. E. & Wolf-Gladrow, D. 2001. *CO2 in seawater: equilibrium, kinetics, isotopes,* Amsterdam, Elsevier.

Appendix

At the end of the MSc thesis, a stay at the CARMABI foundation on Curacao was conducted. First try-outs with microsensors on planktonic foraminifer were conducted. A short summary on the fieldtrip follows.

Planktonic foraminifera were collected at Piscadera Bay in Curacao between 6th of February and 10th of March by scuba diving. For the collection of planktonic species, the set-up described by Heine (1986) was used. Scuba divers were attached to a boat in the open ocean (2-12km distance from the coast) and they captured single individuals with glass bottles. Planktonic species were first identified, then kept in the same glass bottles with the ambient seawater in it and fed daily with one brine shrimp (*Artemia*). Afterwards they were stored in a temperature-stable incubator at 26°C and were regularly checked and if they looked healthy (well visible spines, floating), one individual was selected and placed on a nylon mesh fixed on a tube within a petridish (Fig. 13) in filtered (0.7μm) seawater from the reef of Piscadera Bay, Curacao.

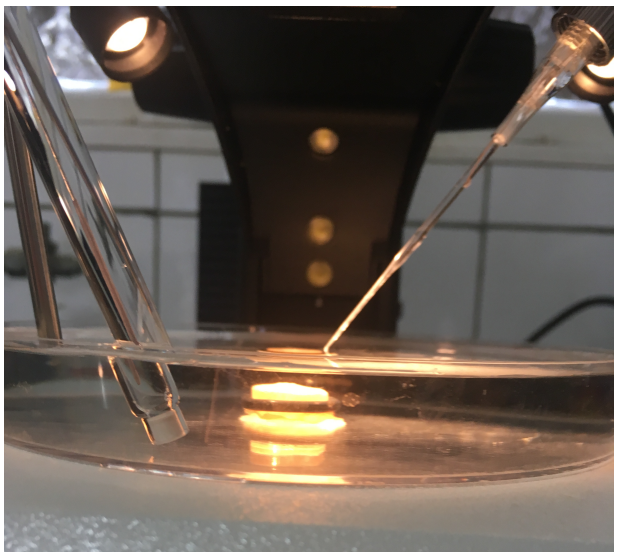

Figure 13. The set up used during microsensor studies. On the left the reference electrode is visible, in the middle the tube with the nylon mesh on which the foraminifera was place. Furthermore, the microsensor is visible in the picture. The whole set up was placed under a microscope.

Salinity (36.00 ± 0.22‰) and water temperature of the filtered seawater (24.53 ± 0.51°C) and the pCO_2 concentration of the room (857.50 ± 309.12 ppm) was determined.

Microsensor measurements were conducted with 10 μm Unisense pH-electrodes. The microelectrodes were calibrated using a three point calibration with pH buffers of pH 4, 7 and 10 at the beginning and end of each measurement. If the calibration points were not exactly

the same at the beginning and the end of the experiment (due to drifts of the pH sensor accuary), a linear interpolation between the points led to the final calibration curve for the micorsensors. All measurements were recorded with the software PICO log in mV and afterwards converted to a pH scale using the calibration. The sensor tip was placed as close as possible to the shell of the foraminifera and pictures were taken regularly. If the foraminifera moved away, the microsensor tip was moved closer to the shell again.

The first measurement was conducted on the planktonic species *Globigerinella siphonifera*.

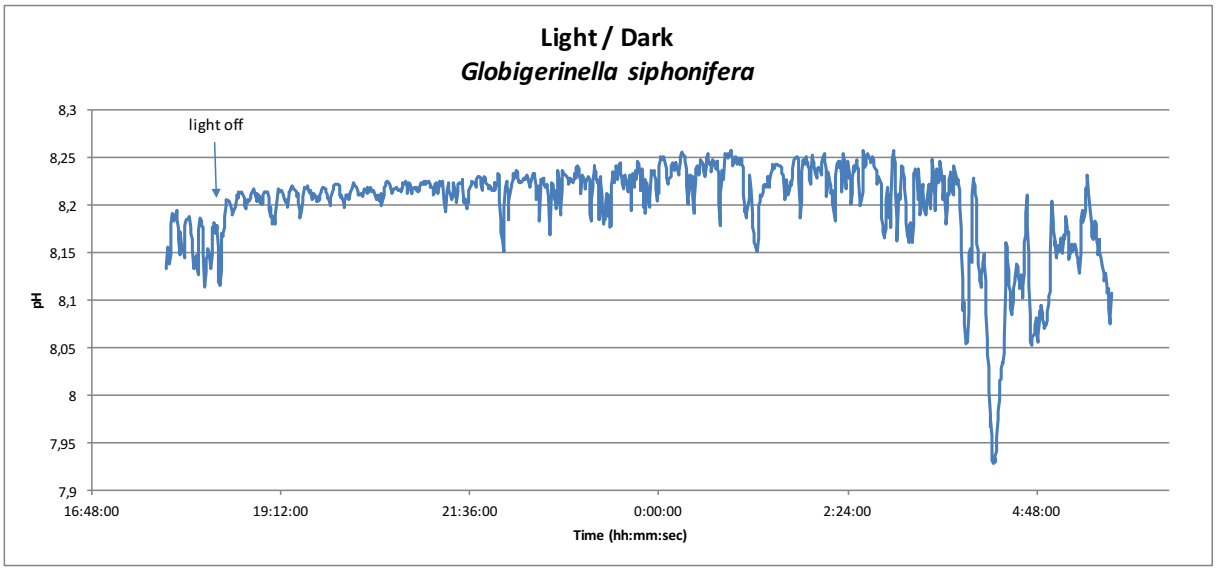

Figure 14. Measurement conducted on Globigerinella siphonifera over approx. 8 hours. After the first hour light was turned off (following the natural daily cycle).

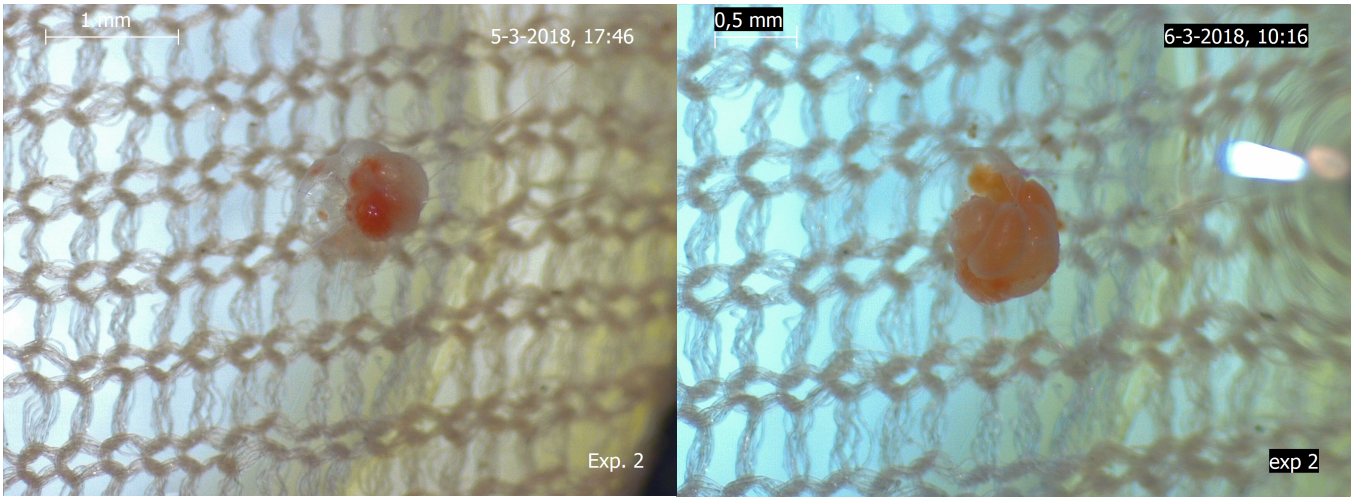

Figure 15. Picture on the left at the beginning of the experiment. Picture on the right was taken the next morning, after the experiment was finished.

First light was turned on and after approx. 45 minutes it was turned off and pH was measured constantly. In the darkness pH was expected to decrease, due to respiration of CO_2, as was also found in another study for the planktonic species *Orbulina universa* (Rink et al., 1998, Köhler-Rink and Kühl, 2005). However, against our expectations pH increased after the light was turned off (Fig. 14). This was most probably due to a temperature decrease without the light source. Furthermore, a strong negative peak appeared in the measurements after about 10 hours of darkness. Pictures were taken at the beginning and at the end of the experiment, but it is not clearly visible whether the foraminifera calcified in this period (Fig. 15).
Other conducted measurements resulted in similar signals.