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Biometry and dissolution features of the benthic foraminifer *Ammonia aomoriensis* at high pCO_2

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ABSTRACT: Culturing experiments were performed with the benthic foraminifer *Ammonia aomoriensis* from Flensburg Fjord, western Baltic Sea. The experiments simulated a projected rise in atmospheric CO_2 concentrations. We exposed specimens to 5 seawater pCO_2 levels ranging from 618 µatm (pH 7.9) to 3130 µatm (pH 7.2) for 6 wk. Growth rates and mortality differed significantly among pCO_2 treatments. The highest increase of mean test diameter (19%) was observed at 618 µatm. At partial pressures >1829 µatm, the mean test diameter was observed to decrease, by up to 22% at 3130 µatm. At pCO_2 levels of 618 and 751 µatm, *A. aomoriensis* tests were found intact after the experiment. The outer chambers of specimens incubated at 929 and 1829 µatm were severely damaged by corrosion. Visual inspection of specimens incubated at 3130 µatm revealed wall dissolution of all outer chambers, only their inner organic lining stayed intact. Our results demonstrate that pCO_2 values of ≥929 µatm in Baltic Sea waters cause reduced growth of *A. aomoriensis* and lead to shell dissolution. The bottom waters in Flensburg Fjord and adjacent areas regularly experience pCO_2 levels in this range during summer and fall. Increasing atmospheric CO_2 concentrations are likely to extend and intensify these periods of undersaturation. This may eventually slow down calcification in *A. aomoriensis* to the extent that net carbonate precipitation terminates. The possible disappearance of this species from the Baltic Sea and other areas prone to seasonal undersaturation would likely cause significant shifts in shallow-water benthic ecosystems in the near future.

KEY WORDS: Benthic foraminifera · Biometry · Test dissolution · Carbon dioxide · Baltic Sea

INTRODUCTION

The rise in atmospheric CO_2 concentrations has caused an increase in seawater pCO_2 over the past 250 yr (Takahashi 2004, Solomon et al. 2007). Surface ocean waters have taken up ~30% of anthropogenic CO_2 (Sabine et al. 2004, Khatiwala et al. 2009), causing a reduction in ocean pH and carbonate ion concentration (Orr et al. 2005, Cao & Caldeira, 2008). In response to this acidification, the calcium carbonate saturation state for calcite and aragonite will be lowered to half their present-day values by 2300 (Feely et al. 2004, Caldeira & Wickett 2005). This reduced saturation state and reduction in carbonate ion concentration is expected to negatively affect shell and skeleton construction by calcifying organisms (e.g. Erez 2003).

Benthic foraminifera are the most diverse group of hard-shelled protists. They live at the sediment–water interface, or within the sediments down to >12 cm depth (Corliss 1985). Model calculations have inferred that benthic foraminifera account for from 5 to 30% of carbonate production in shallow waters (Wefer 1976, Langer 2008). The benthic foraminiferal fauna is estimated to precipitate 0.2 Gt $CaCO_3$ per year on a global scale (Langer et al. 1997, Langer 2008), which amounts

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to about one-third of the production by planktonic foraminifers (Schiebel 2002).

In addition to CO_2-induced ocean acidification, anthropogenic eutrophication by river and groundwater discharge and by atmospheric deposition can lead to changes in carbonate chemistry, especially in coastal marine environments such as the Baltic Sea (Rosenberg 1985, Conley et al. 2007, Levin et al. 2009, Borges & Gypens 2010, Cossellu & Nordberg 2010, Zhang et al. 2010). In comparison to the open ocean, the Baltic Sea exhibits lower salinities, lower $[CO_3^{2-}]$ and consequently lower calcium carbonate saturation states (Ω). In the western Baltic Sea, seasonal effects are superimposed (Hansen et al. 1999). Vertical stratification, enhanced microbial activity and the ensuing consumption of dissolved oxygen by the decay of particulate organic matter causes hypoxic conditions in the bottom water and therefore strong seasonally varying pCO_2 values over the year (Diaz & Rosenberg 2008, Conley et al. 2009, Thomsen et al. 2010). In response to low Ω and seasonal acidification, a reduced calcification of foraminifera is expected in Flensburg Fjord (Polovodova et al. 2009).

An increasing number of field and laboratory studies have shown that many calcareous organisms have lower calcification rates under simulated ocean acidification (e.g. Riebesell et al. 2000, Langdon & Atkinson 2005, Orr et al. 2005, Moy et al. 2009, Thomsen & Melzner 2010). There is also evidence that planktonic foraminifers precipitate thinner test walls at reduced carbonate ion concentrations and higher atmospheric CO_2 levels (Spero et al. 1997, Bijma et al. 1999, Moy et al. 2009). A recent study by Kuroyanagi et al. (2009) investigated growth rates of the tropical, symbiont-bearing foraminifer *Marginopora kudakajimensis* during long-term incubation at 4 different pH_{NBS} (National Bureau of Standards pH) levels between 8.3 and 7.7. Their results indicated that growth rate, shell weight, and the number of newly added chambers decreased with a lowering of the pH. A further culturing experiment with the benthic foraminifer *Elphidium williamsoni* indicated the formation of significantly thinner chamber walls at a pH of 7.6 (Allison et al. 2010). Specimens of the boreal shallow-water species *Ammonia tepida* were cultured under atmospheric CO_2 concentrations of 120 µatm (pH 8.4) and 2000 µatm (pH 7.5; Dissard et al. 2009). Surprisingly, the specimens still calcified at concentrations <2000 µatm. This was in contrast to earlier experiments with living *A. beccarii* from Isle de Yeu, France. In the Ile de Yeu study, growth ceased and dissolution of the tests started at the same pH_{NBS} of 7.5 (Le Cadre et al. 2003).

The consequences of future elevated atmospheric CO_2 concentrations for benthic foraminiferal calcification in shallow waters are thus not sufficiently studied. The purpose of this study was to investigate the calcification response of the benthic foraminiferal *Ammonia aomoriensis* from the western Baltic Sea to different seawater pCO_2 levels.

MATERIALS AND METHODS

Sampling and cultivation of foraminifera. Living *Ammonia aomoriensis* specimens were collected from Flensburg Fjord, western Baltic Sea (54° 48.082' N, 9° 53.069' E, 13 m water depth), in June 2009. This location is situated 0.316 n miles to the northwest of Station PF16-26, where, in June 2006, *A. aomoriensis* was reported (as *A. beccarii*) as dominating the living assemblages (Polovodova et al. 2009). The bottom sediment is a silty fine sand. We used a Mini Corer (inner diameter 100 mm; Kuhn & Dunker 1994), deploying it from RV 'Littorina'. Altogether, 4 cores were taken. The first 2 cores were used for determination of carbonate system parameters and also to serve as backup material. Once aboard, the uppermost 1 cm of the third core was gently washed with seawater of 20 psu through a 63 µm mesh. The residue was kept in 300 ml Kautex wide-neck containers with seawater. The bottles were covered with Parafilm to avoid excess evaporation, and were then aerated and stored at 20°C as stock cultures. The cultures were exposed to a 12 h light:12 h dark (12:12 L/D) cycle. Foraminifera were fed with 200 µl of a living algae mixture containing *Nannochloropsis oculata*, *Phaeodactylum tricornutum* and *Chlorella* (DT's Premium Blend) once a week. During feeding, the air pumps were switched off for 1.5 to 2 h to allow the algae to settle, and to facilitate successful feeding of the foraminifera.

Occurrence and identification of *Ammonia* species from Flensburg Fjord. In the western Baltic Sea, *Ammonia* spp. is common at 4 to 14 m water depth. It lives in muddy sands under brackish conditions with salinities ranging from 15 to 23 psu (Rottgardt 1952, Lutze 1965, Nikulina et al. 2008), and is found up to 6 cm deep in the sediment (Lutze 1987). Initially, *Ammonia* spp. from Kiel Bight and adjacent fjords were identified as *Ammonia beccarii* (Linné 1758), applying a broad understanding of this taxon (Schnitker 1974). *Ammonia* spp. from European marginal seas have in fact mostly been identified as *Ammonia beccarii* or *Ammonia tepida* (Haake 1962, Lutze 1965, Murray 1991, Debenay et al. 1998, Bouchet et al. 2007, Pascal et al. 2008). However, the diameter of our specimens from Flensburg Fjord was about 1.5 times larger than that of *Ammonia tepida* lectotypes from Puerto Rico (Cushman 1926, Hayward et al. 2003). The Flensburg Fjord specimens commonly had 9 chambers in the last whorl, while *Ammonia tepida* lectotypes showed only 7 chambers, and the outline of Flensburg

Fjord tests was less lobular than those from Puerto Rico. Topotypes of *Ammonia beccarii* from Rimini Beach, Italy, were much flatter, had 14 to 15 chambers in the last whorl, and showed a distinct ornamentation on both spiral and umbilical sides (Hayward et al. 2004). Such ornamentation is lacking in our specimens from Flensburg Fjord. *Ammonia* specimens from Flensburg and Kiel Fjord were almost identical in shape and morphology (Polovodova & Schönfeld, 2008). A molecular identification of *Ammonia* specimens collected in the Kiel Fjord with rDNA sequences revealed that they belong to the phylotype T6, which, based on morphological characters, was referred to the Pliocene species *Ammonia aomoriensis* (Asano 1951), which is likely to be extant (Hayward et al. 2004, Schweizer et al. 2010). The adjacent occurrence in the same marginal sea and the strong morphological similarity with specimens from Kiel Fjord suggests that *Ammonia* from Flensburg Fjord also represents the species *A. aomoriensis*. Ongoing molecular phylogenetic analysis by M. Schweizer, University of Edinburgh, is expected to provide more information about molecular identification of Flensburg Fjord *Ammonia* based on rDNA sequences.

Preparation of foraminifera. Living specimens were picked with a fine brush from the stock-cultures under a Wild M3C dissecting microscope. All individuals of *Ammonia aomoriensis* were divided into 3 groups of distinctively different behavior: small and 'active' young specimens (size class 150 to 250 µm), larger and 'active' young specimens (size class 250 to 350 µm) and 'inactive' 'old' specimens of >200 µm in diameter. The specimens were identified as being alive by their yellow cytoplasm content. Additionally, selected and presumably living specimens of 2 different size classes (150 to 250 µm and 250 to 350 µm) were aligned in a Petri dish and left for half an hour. This is an infallible method for distinguishing active from inactive individuals. Only those individuals that showed a lateral movement of at least 3 mm were considered active specimens. Larger, inactive specimens of >200 µm in diameter from both size classes showed a lateral movement of 0.5 to 1 mm only. These inactive specimens were adults, previously considered inappropriate for culturing (Barras et al. 2009).

All specimens, when we selected them, had the same stress condition, independent of activity level or size. We considered specimen movement — an individual and active response to disturbance of habitat — to be indicative of current physical condition, and on this basis we presumed physical condition to be the same for all specimens showing the same response behavior.

After their classification, the selected specimens were exposed to calcein-stained seawater (4 mg l^{-1}) for 2 wk (Bernhard et al. 2004, Barras et al. 2009) and then placed into 300 ml transparent polycarbonate culture vessels, 30 specimens to a vessel. Each vessel contained 10 small active, 10 larger active and 10 large inactive specimens (Fig. 1). This was done at 5 different pCO_2 levels and 3 replicates for each pCO_2 level and each size fraction.

Specimens were kept individually in 1 mm deep recruitment pits of 7 mm diameter, which were drilled into the base plate of the vessels. The recruitment pits were not enclosed, so that the specimens could move and seek shelter inside the pits. The basic idea was that the recruitment pits would help us locate the specimens for monitoring. We observed that most specimens moved around only within their pits, with only very few leaving the pits. (It might have been better to have had deeper or more enclosed pits, but this in turn might have caused a more unstable pCO_2 gradient in each pit.) A small amount (0.43 g) of carbonate-free quartz (>97 % SiO_2) was dispersed in equal amounts among the pits with the intention of better mimicking the natural habitat of *Ammonia aomoriensis*. The microenvironment along the base plate of the vessel appeared to be less attractive to the specimens, since most of them stayed in their pit.

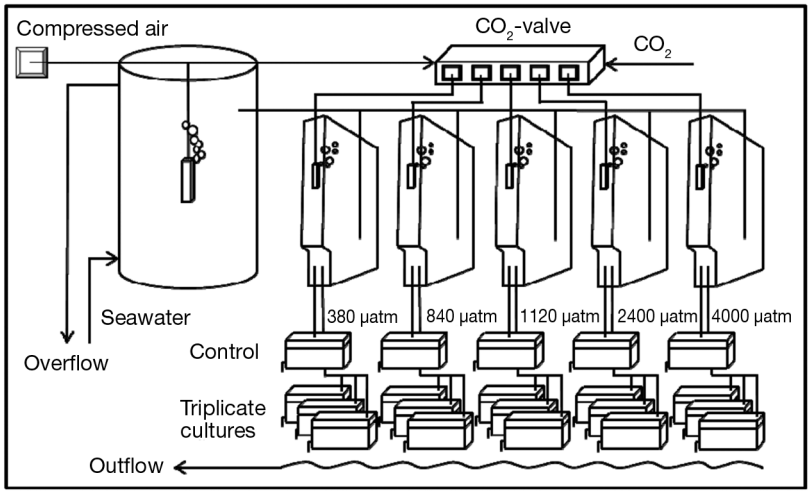

Fig. 1. *Ammonia aomoriensis.* Setup of culturing system. For explanation, see 'Experimental setup'

Experimental setup. Culturing of *Ammonia aomoriensis* was performed in a flow-through system following the concept of Hintz et al. (2004) (Fig. 1). The culture vessels were flushed with cartridge-filtered (25 µm) and UV-sterilized seawater from Kiel Fjord. In order to monitor the carbonate system, pH according to the National Bureau of Standards pH-scale (pH_{NBS}), total alkalinity

(A_T), salinity, temperature and phosphate concentrations (PO_4^{3-}) were measured continuously in the flow-through system. The seawater was enriched with oxygen in a 30 l reservoir bin, and subsequently conditioned in five 5 l compact jerrycans with CO_2-enriched compressed air at partial pressures of 380, 840, 1120, 2400 and 4000 µatm. The pCO_2 range from 380 to 1120 µatm corresponded to values recorded in Flensburg Fjord; the 2 higher levels were meant to simulate future scenarios of 2400 and 4000 µatm. The preconditioned seawater from each jerrycan flowed through 4 culture vessels. Three vessels contained living foraminifera as triplicate experiments at the same pCO_2 exposure, and 1 vessel was left barren as a control for hydro-chemical monitoring. To replace the water in the aquaria 1.4 times h^{-1}, the flow rate was adjusted to 0.16 ml s^{-1}. The overflow seeped through the fissure between lid and vessel, draining off to a sink. Food was added every second day to the vessels containing foraminifera as 100 µl DT's Premium Blend algae mixture. The experiment lasted 6 wk.

Population dynamics and biometry. The aquarium-like culture vessels permitted monitoring of the individuals throughout the experiment. They were examined weekly under a dissecting microscope, and their presence, shape and behavior noted. As the removal of culture vessels from the experimental setup for examination induced some disturbance, we refrained from surveillance at shorter intervals. Using an eyepiece reticle on the dissecting microscope, we measured the size of all specimens weekly in their recruitment pits. These measurements had to be made through the water column in the culture vessels. During these measurements, temperature was held stable by placing the culture vessel in a water bath with crushed ice, and monitoring the temperature.

Because of a working distance of >50 mm, we could use only 40× magnification, which resulted in an error of ±12.5 µm (maximum distance to the next scale unit) in the size measurements. This is approx. ±4 to ±7% of the average diameter (from 180 to 280 µm) of the examined specimens. The overall test diameter of specimens changed during the experiment. Size differences were calculated by measuring the test diameter at the beginning and at the end of the 6 wk experiment. In addition to diameter measurements, we determined the number of new chambers formed during the incubation period. We did this at the end of the experiment by examining the specimens (according to Dissard et al. 2009) under an inverted fluorescence microscope (Zeiss Axiovert 100, wavelength: 530 nm). After the experiment, all individuals were stained with Rose Bengal to assess whether they still contained cytoplasm (indicating living specimens) or not (dead specimens).

Water chemistry. pH, as well as alkalinity, salinity, temperature and phosphate concentrations, were measured and compared in the culture vessels and controls. As an additional control we measured all parameters in the 30 l reservoir bin, in the five 5 l compact jerrycans and in the sink. pH_{NBS}, temperature and salinity were monitored every second day in the setup. We used a WTW 340i pH analyzer to measure pH and water temperature. The pH analyzer was calibrated with standard buffer solutions of pH 4.01, 7.00 and 10.00 (WTW standard, DIN/NIST buffers L7A). Precision was ±0.01 for pH and ±0.1°C for temperature. For salinity measurements, a WTW Cond 315i salinometer with a precision of ±0.1 psu was used.

In order to calculate carbonate system parameters with CO_2SYS software, the phosphate concentration was measured weekly (Lewis & Wallace 1998). A 10 ml water sample was passed through a 0.2 µm filter and was measured colorimetrically in a spectrophotometer (U 2000, Hitachi-Europe) at a wavelength of 882 nm according to Koroleff (1983). The precision of the phosphate measurements was ±0.2 μmol l^{-1}.

Samples for analysis of total alkalinity (A_T) were sterile-filtered (0.2 µm pore size) and determined through potentiometric titration (Dickson, 1981) in a Metrohm Tiamo automatic titration device. The precision of the alkalinity measurements was 2 μmol kg^{-1}. The carbonate system parameters pCO_2 and Ω calcite values were calculated from measured A_T, pH, phosphate, temperature and salinity using the CO_2SYS software (Lewis & Wallace 1998). The equilibrium constants of Mehrbach et al. (1973), as refitted by Dickson & Millero (1987), were chosen.

Table 1. *Ammonia aomoriensis*. Carbonate chemistry of bottom water from sampling station FF3 from Flensburg Fjord (54° 48.082′ N, 9° 53.069′ E), showing duplicate average values of salinity, temperature, total alkalinity (A_T) and the pH according to the National Bureau of Standards pH-scale (pH_{NBS}). The precision of the alkalinity measurements was 100 µmol kg^{-1}. Carbon dioxide partial pressure (pCO_2) and Omega of calcite saturation state (Ω_{ca}) were calculated (cal) with the CO_2Sys program (Lewis & Wallace 1998) from measured A_T, pH_{NBS}, temperature and salinity

Date (d/mo/yr)	Salinity	Temp. (°C)	A_T (µmol kg^{-1})	pH_{NBS}	pCO_2 cal (µatm)	Ω_{ca} cal
03/06/09	20.2	10.3	2125.5 ± 87.6	7.83	727.1	1.35
18/08/09	19.8	15.3	2239.1 ± 28.0	7.47	1863.3	0.85
20/10/09	21.6	11.9	2465.7 ± 31.4	7.31	2873.8	0.60
07/12/09	20.9	8.8	2174.0 ± 64.1	7.81	769.1	1.42
15/02/10	16.9	−0.4	1804.6 ± 200.7	7.94	465.1	1.05
19/04/10	18.8	5.6	2374.9 ± 90.8	7.94	493.0	2.01

Near-bottom water samples were taken from Flensburg Fjord in order to determine salinity, temperature, pH and alkalinity in the natural habitat of *Ammonia aomoriensis*. The range of reproducibility of pH measurements from Flensburg Fjord bottom-water samples, collected bi-monthly from June 2009 to April 2010, was from 0.04 to 0.1. Carbonate system parameters were calculated from measurements of pH_{NBS} and alkalinity. Monitoring results will be reported elsewhere, but we refer to the pCO_2 values and their seasonal range in the present study (Table 1).

Preparation for scanning electron microscopy. At the end of the experiment, the foraminifers were removed from the culture vessels using a fine brush and were transferred to Eppendorf-type micro centrifuge tubes. Fixation was accomplished in a solution of 2 g Rose Bengal in 1 l ethanol (98%, technical quality) for 24 h. Finally, intact specimens were air-dried, prepared with an Emitech K550 (Au+Pd) sputter coater and photographed with a scanning electronic microscope (SEM; Cam Scan-CS-44).

Statistics. Changes in test diameter (see Fig. 3B) were analyzed by linear regression ($f = b + ax$) using SIGMA PLOT 10. Regression lines present Pearson correlation with confidence bands, which exhibit 95% CI and correlation coefficient R^2 for the fitted line. The error in the regression equations is ±1 SE of the mean.

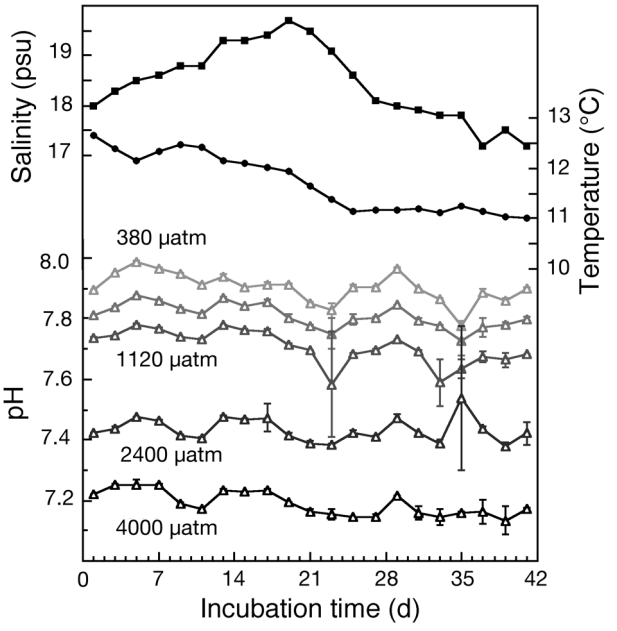

Fig. 2. *Ammonia aomoriensis*. Water chemistry during the experiment. Temperature and salinity are mean values of all measurements taken in each culture vessel flushed with different seawater pCO_2 levels as in Table 1. pH: means ± SD, n = 3

RESULTS

Water chemistry

Salinity ranged from 17 to 19.5 psu (mean ± SE = 18.4 ± 0.8) during the experimental period (Fig. 2, Table 2). Mean seawater temperature in the culture vessels decreased steadily over the course of the experiment from 12.6 to 11°C. The culture vessels were flushed with seawater, which was taken from Kiel Fjord with our aquarium system. Salinity and temperature of seawater changed seasonally (Thomsen et al. 2010). The fluctuations of these parameters during the experimental period therefore correspond to the natural variability in *Ammonia aomoriensis* habitat.

pH values (mean ±1 SE) varied according to seawater pCO_2 from 7.9 ± 0.05 to 7.2 ±0.04. They were

Table 2. *Ammonia aomoriensis*. Carbonate chemistry of culture media, means ±SD (n = 3) of several variables for 5 pCO_2 levels. Controls are mean values of all measurements made during the 6 wk incubation. pCO_2, total carbon (C_T), and Omega of calcite saturation state (Ω_{ca}) were calculated (cal) with the CO_2Sys program (Lewis & Wallace 1998) from measured A_T, pH_{NBS}, PO_4^{3-}, temperature and salinity

pCO_2 baseline level (µatm)	pCO_2 cal (µatm)	Salinity	Temp. (°C)	pH_{NBS}	A_T (µmol kg^{-1})	C_T cal (µmol kg^{-1})	Ω_{ca} cal	PO_4^{3-} (µmol l^{-1})
380	617.9 ± 8.5	18.4 ± 0.8	11.8 ± 0.6	7.90 ± 0.05	2040.1 ± 20.7	1980.2 ± 31.8	1.66 ± 0.25	0.99 ± 0.33
380_control	610.1 ± 94.0	18.4 ± 0.8	11.7 ± 0.6	7.91 ± 0.04	2043.1 ± 20.1	1983.1 ± 29.2	1.67 ± 0.22	0.99 ± 0.31
840	751.1 ± 22.8	18.4 ± 0.8	11.6 ± 0.6	7.81 ± 0.04	2039.0 ± 17.4	1999.5 ± 22.3	1.38 ± 0.16	1.02 ± 0.32
840_control	734.7 ± 67.8	18.4 ± 0.8	11.6 ± 0.7	7.81 ± 0.04	2036.0 ± 16.7	1996.4 ± 21.7	1.39 ± 0.14	1.04 ± 0.29
1120	929.1 ± 23.4	18.4 ± 0.8	11.7 ± 0.6	7.71 ± 0.06	2035.2 ± 19.8	2014.4 ± 23.2	1.14 ± 0.13	1.01 ± 0.33
1120_control	953.9 ± 147.4	18.4 ± 0.8	11.6 ± 0.5	7.71 ± 0.04	2034.0 ± 14.6	2018.0 ± 19.2	1.12 ± 0.15	1.06 ± 0.32
2400	1829.2 ± 33.5	18.4 ± 0.8	11.6 ± 0.6	7.43 ± 0.04	2036.2 ± 21.5	2075.2 ± 20.4	0.64 ± 0.08	1.01 ± 0.30
2400_control	1891.2 ± 227.0	18.4 ± 0.8	11.6 ± 0.6	7.42 ± 0.04	2042.0 ± 14.6	2095.7 ± 21.0	0.59 ± 0.06	1.00 ± 0.30
4000	3130.2 ± 33.6	18.4 ± 0.8	11.9 ± 0.5	7.19 ± 0.04	2039.7 ± 19.2	2156.2 ± 24.5	0.37 ± 0.04	1.00 ± 0.33
4000_control	3158.6 ± 235.1	18.4 ± 0.8	11.8 ± 0.5	7.18 ± 0.05	2036.9 ± 17.8	2159.8 ± 23.7	0.36 ± 0.03	0.96 ± 0.37

reasonably stable during the whole incubation time and did not mirror variations in salinity and temperature (Fig. 2). Maxima and minima in mean values were caused by a few exceptionally high and low values in single replicates, which may represent measurement errors. Alkalinity and phosphate concentration also showed no significant change over the course of the experiment (Table 2). No systematic offsets or significant differences among experimental and control vessels were detected. While respiration and degradation processes are likely to be enhanced when food is added to the vessels, we saw no significant differences of measured or calculated parameters between controls and cultures. This suggests that the amount of food added was too small to change the abiotic conditions in the culture vessels at the given flow rates.

The calculated pCO_2 in the culture vessels differed significantly from the pCO_2 baseline level in the compressed air (Table 2). For instance, in the jerrycan bubbled with compressed air without CO_2 addition, which should yield the ambient atmospheric partial pressure of 380 µatm, the measured value was 618 µatm. This was most likely due to a higher CO_2 concentration in subsurface waters of Kiel Fjord at the seawater system intake caused by seasonal phenomena, such as upwelling of hypoxic and hypercapnic waters (Thomsen et al. 2010). At higher pCO_2 levels, the measured values in the culture vessels were 11 to 24% lower than the target values in the CO_2-charged compressed air (Table 2). The difference probably accounts for outgassing in the culture vessels due to the slow percolation rate. In the following, we refer to the pCO_2 values that were calculated from actually measured hydrochemical parameters in the culture vessels and not to the pre-adjusted values in the CO_2-enriched air (Table 2).

The Ω values (mean ± 1SE) for calcite ranged from 1.66 ± 0.25 at a pCO_2 of 618 µatm to 0.37 ± 0.04 at a pCO_2 of 3130 µatm. The values <1.0 indicate carbonate dissolution at partial pressures above 929 µatm under the present settings of temperature and salinity.

The carbonate system parameter pCO_2 cal and Ω_{Ca} cal, as calculated from measured pH_{NBS} and A_T values, varied in the near-bottom water at the sampling site in Flensburg Fjord from 2874 µatm (pH 7.3) in October 2009 to 465 µatm (pH 7.9) in February 2010 (Table 1). In August and October 2009, Ω values for calcite were temporarily <1.0 (0.85 and 0.6, respectively). Therefore, our experiment covers the entire seasonal pCO_2 variability in the *A. aomoriensis* habitat in Flensburg Fjord, even though we did not capture the seawater pCO_2 level in February and April 2010 when the pCO_2 in Flensburg Fjord was ~130 µatm lower than at the lowest partial pressure in our experiment.

Test diameters

All specimens, whether inactive or active, were alive at the beginning of the experiment. They grew during the incubation, especially at low pCO_2 values. Active specimens from the 150 to 250 µm fraction displayed an increase of 19% in diameter at a pCO_2 of 618 µatm, whereas mean diameter of active specimens from the 250 to 350 µm fraction increased by only 11% (Fig. 3A). In comparison to active specimens, the mean diameter of the large inactive specimens (>200 µm) increased by only 2% at a pCO_2 of 618 µatm during the course of the experiment (Fig. 3A).

The growth of specimens from the 150 to 250 µm fraction differed significantly, depending on pCO_2 treatment (Fig. 3B). The greatest increase in mean shell diameter of 35 µm was observed at the lowest pCO_2 level of 618 µatm. At pCO_2 levels of 751 µatm and 929 µatm, the mean diameter of *Ammonia aomoriensis* increased by only 29 and 13 µm, respectively. At a pCO_2 of 1829 and 3130 µatm, the test diameter was reduced by 5 and 41 µm due to test corrosion.

The shell diameter of active specimens from the 250 to 350 µm fraction differed also according to pCO_2 level (Fig. 3B). At a pCO_2 of 618 µatm, the mean shell diameter increased by 29 µm. The growth rate was highest at a pCO_2 of 751 µatm, where the increase was 39 µm. *Ammonia aomoriensis* displayed a reduced growth under higher pCO_2 levels, viz. from 929 to 3139 µatm. While the mean shell diameter increased by 29 µm at a pCO_2 of 929 µatm, the specimens displayed barely any change in size at a pCO_2 of 1829 µatm during the 6 wk incubation period. At a seawater pCO_2 of 3130 µatm the average shell diameter was reduced by 23 µm.

Inactive specimens from the >200 µm fraction showed only a slight change of test diameter at a pCO_2 of from 618 to 1829 µatm (Fig. 3B). The lowest increase (6 µm) was observed at a pCO_2 of 618 µatm, followed by an increase of 21 µm at a pCO_2 of 751 µatm and 13 µm at 929 µatm. Like active specimens, the shell diameter barely changed at a pCO_2 of 1829 µatm and, on average, decreased by 48 µm at a pCO_2 of 3130 µatm.

Loss and mortality rates

During the 6 wk incubation period, some *Ammonia aomoriensis* specimens disappeared between weekly surveillance periods (Fig. 4). On these occasions the entire culture vessel was thoroughly screened twice for lost specimens. However, the missing individuals had neither moved out of their recruitment pits, nor had they crawled upwards on the sidewalls of the

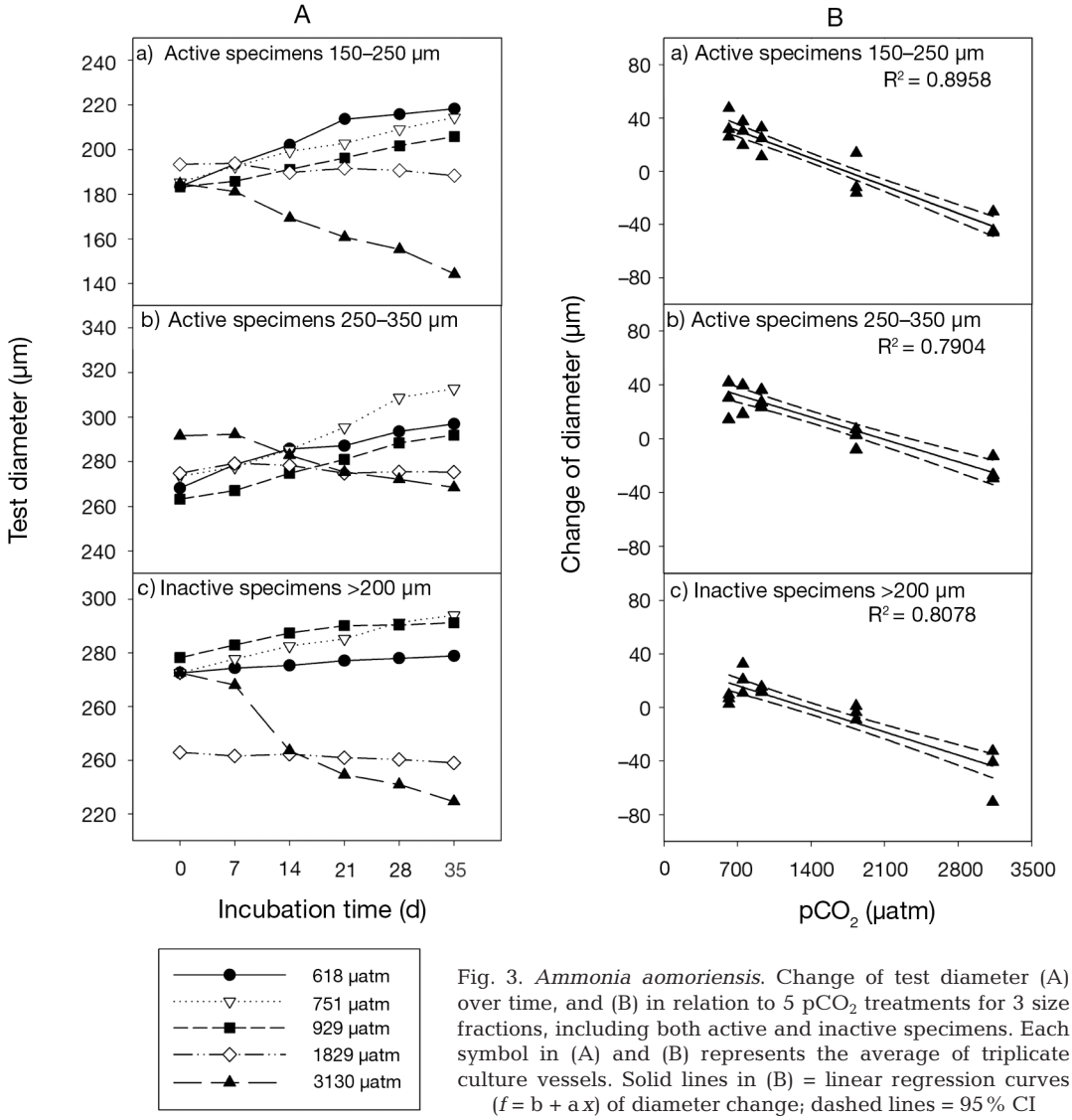

Fig. 3. *Ammonia aomoriensis*. Change of test diameter (A) over time, and (B) in relation to 5 pCO_2 treatments for 3 size fractions, including both active and inactive specimens. Each symbol in (A) and (B) represents the average of triplicate culture vessels. Solid lines in (B) = linear regression curves ($f = b + a\,x$) of diameter change; dashed lines = 95% CI

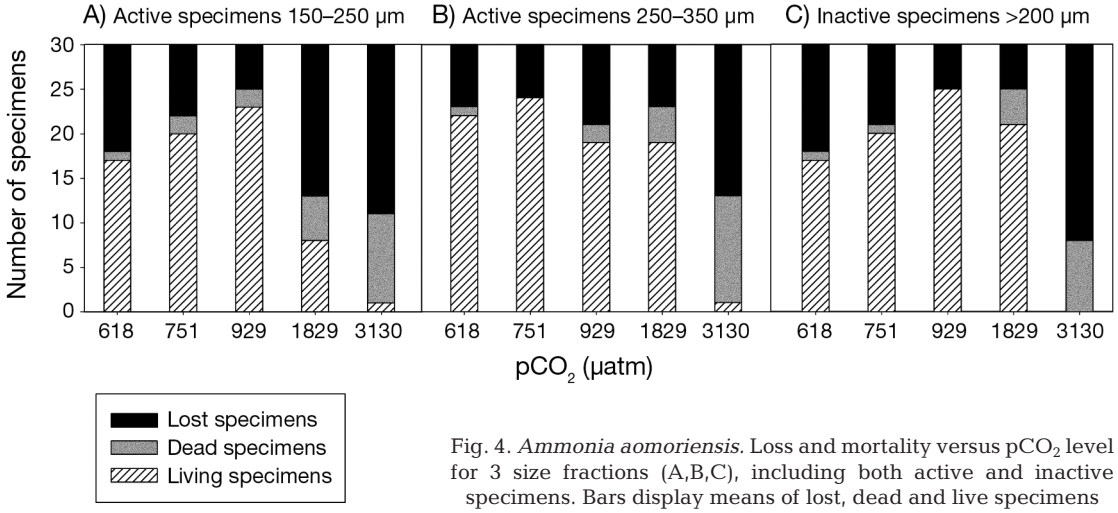

Fig. 4. *Ammonia aomoriensis*. Loss and mortality versus pCO_2 level for 3 size fractions (A,B,C), including both active and inactive specimens. Bars display means of lost, dead and live specimens

vessels (e.g. Lee & Anderson 1993). Nor were encystation or clustering of juveniles around the mother individual observed (e.g. Lehmann 2000, Heinz et al. 2005). The physical disturbance during removal of vessel, surveillance and sampling might have played a role in this occurrence. Allison et al. (2010), moreover, have described the possibility of flotation and escape of specimens attached to air bubbles with seawater outflow from the cultivation chambers. The inner organic lining, which is light and floats easily, may also be involved in escape by flotation. In our setup, however, air bubbles were trapped under the lid of the vessels, thus eliminating this option. It remains possible that some of the specimens were lost during sampling.

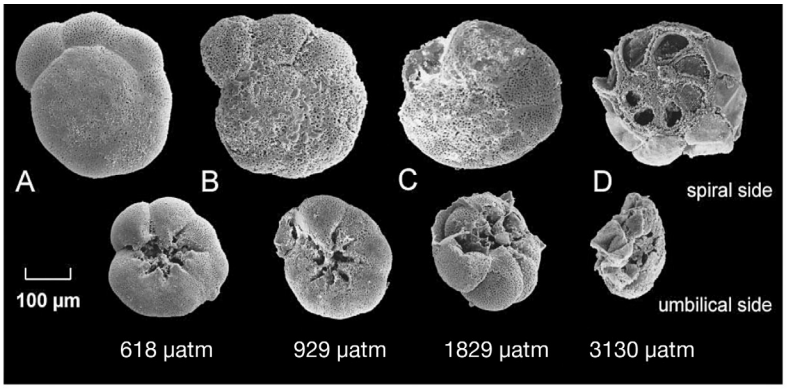

Fig. 5. *Ammonia aomoriensis.* SEM images depicting different stages of dissolution on spiral and umbilical sides of the test at various pCO_2 levels (A to D)

The loss resulted in no significant difference between the active specimens of size fractions 150 to 250 µm, 250 to 350 µm or inactive specimens >200 µm. In the pCO_2 range of 618 to 929 µatm, the losses of active and inactive specimens during the experimental period averaged from 7 to 11 of the 30 cultured specimens. Significantly higher losses were observed at a pCO_2 of 3130 µatm. From 30 specimens at the beginning of the experiment, the loss of active specimens of the 150 to 250 µm and 250 to 350 µm size fractions averaged 18 specimens. Among the inactive specimens >200 µm, an average of 22 out of 30 individuals were lost during the experiment (Fig. 4).

At the end of the experiment, the foraminifers were picked individually from the recruitment pits under water. Staining of these organisms with Rose Bengal revealed that most individuals incubated from 618 to 1829 µatm pCO_2 survived. Based on staining evidence, an average of 20 active and inactive specimens from each vessel had survived the experiment. However, of the 12 specimens remaining at experiment's end that had been subjected to a pCO_2 of 3130 µatm, an average of 10 contained no living cytoplasm at the end of the incubation and were presumed dead.

SEM observations

The different stages of dissolution during the 6 wk incubation time were revealed by SEM observations (Fig. 5). The tests of *Ammonia aomoriensis* exposed to a pCO_2 of 618 to 751 µatm were fully intact (Fig. 5A). The test walls exhibited a smooth surface, and the pore size and distribution on the shell wall remained unaffected. At pCO_2 levels of 929 and 1829 µatm the last 1 to 3 younger chambers were severely decalcified or destroyed (Fig. 5B,C). The surface of the walls showed fragmentary dissolution of the younger calcite layers, which were left only as scales (Fig. 6A,B). Furthermore, at a seawater pCO_2 of 1829 µatm, we observed the formation of cracks around the pores. (Fig. 6C). On some individuals, at a pCO_2 of 1829 µatm, the SEM examination revealed prograding incisions along the sutures. At a high pCO_2 of 3130 µatm, the tests had become heavily decalcified after 6 wk (Fig. 5D). The tests showed an irregular shape caused by dissolution of the outer chamber walls (Fig. 6D). In all cases, the shell was interrupted and only the inner organic lining remained (Fig. 5D). The interlocular walls were isolated, and their internal calcite layers were separated (Fig. 6D). The remaining interlocular walls gave the tests a star-like appearance (Fig. 5D).

DISCUSSION

Change of test diameter of *Ammonia aomoriensis* under elevated pCO_2

The increase in average test diameter indicated that the individuals had grown during the experiment. Growth was higher among the small young and active specimens than among the large active and inactive adults. At a control pCO_2 of 618 µatm, 78% of the active specimens from the 150 to 250 µm size fraction grew during the experiment, as did 65% of the specimens from the 250 to 350 µm size fraction. Only 44% of inactive specimens of the >200 µm size fraction showed an increase in test diameter, which implied growth by chamber addition during the 6 wk incubation time (Table 3). Substantially more specimens grew in our experiment than during the experiments of Dissard et al. (2009), where ~60% of the specimens kept at a low, pre-industrial CO_2 level added new chambers. With increasing pCO_2, the difference between initial and final test diameter of *Ammonia aomoriensis*

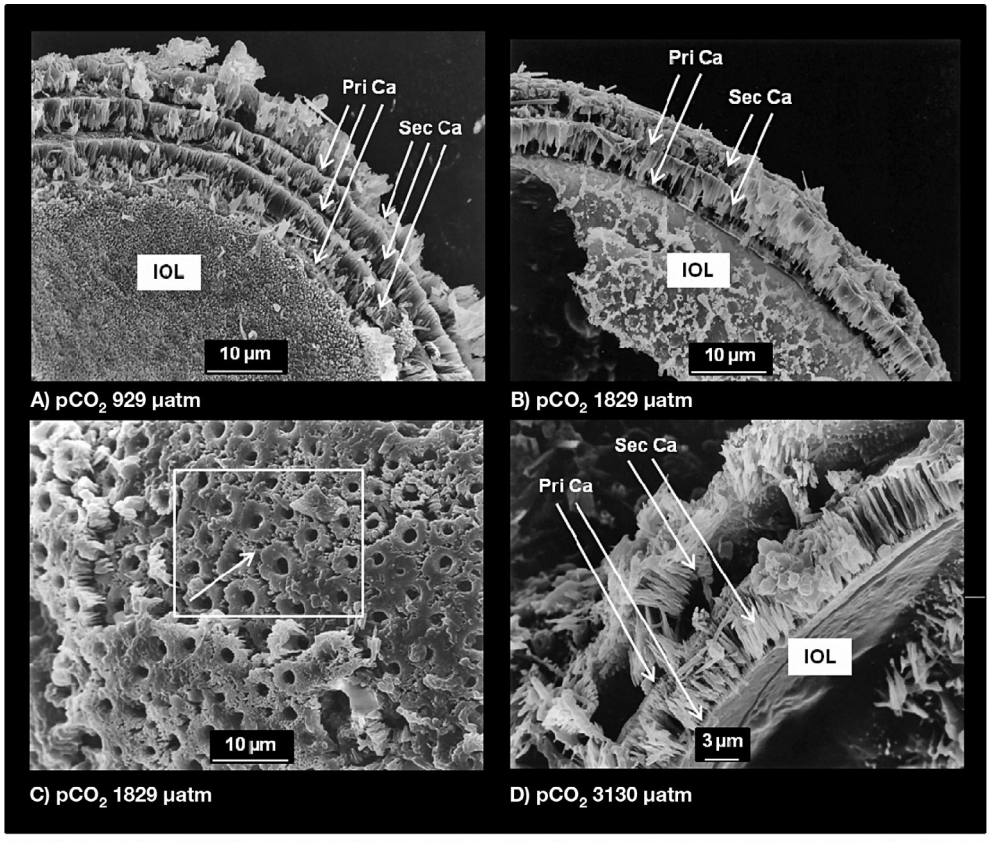

Fig. 6. *Ammonia aomoriensis.* (A,B,D) SEM images showing layers of primary calcite (Pri Ca), secondary calcite (Sec Ca) and inner organic lining (IOL) after 6 wk incubation under elevated pCO_2. (C) Note appearance of cracks at pCO_2 of 1829 µatm

Table 3. *Ammonia aomoriensis.* Newly added chambers from active specimens of 3 size fractions, including both active and inactive specimens, at a control pCO_2 of 618 µatm

Number of specimens	Active specimens 150–250 µm	Active specimens 250–350 µm	Inactive specimens >200 µm
Surviving the experiment	18	23	18
Showing no growth	4	8	10
Grown by 1 chamber	0	1	0
Grown by 2 chambers	6	6	6
Grown by 3 chambers	3	8	2
Grown by 4 chambers	5	0	0
Average chamber addition	**2.3**	**1.6**	**1.0**
Percentage showing growth	**78%**	**65%**	**44%**

decreased. A significant reduction in foraminiferal test diameter was observed at a high pCO_2 of 3130 µatm.

Foraminifera grow by adding new chambers. To assess chamber addition, specimens that had grown during the experiment were examined after the incubation time under a fluorescence microscope. All youngest chambers were stained with calcein (for comparison see Fig. 1A of Allison et al. 2010, p. 88). Since the intensity was hardly distinguishable from the elder part of the test, we could not assess with certainty the number of new chambers that had been formed during the experiment. The newly precipitated calcite of the final chambers contained calcein similar to the walls of the earlier chambers, even though it had not grown in calcein-stained water. We suppose that the calcein was incorporated and stored in vacuoles filled with seawater, from which the calcite for the new chamber wall of the foraminifer was formed. This might explain why the new chambers were fluorescent after the 6 wk incubation time without calcein having been added to the percolating seawater. Another explanation might be that calcein was adsorbed to the organic lining and all calcein-stained chambers were either formed during pre-incubation time or the calcein was re-mobilised from the linings during formation of new chambers. As we observed that foraminifers were stained with calcein before placing them into culture vessels and they had definitely grown during the experiment, this explanation seems less likely. Furthermore, little is known about

the internal dynamics and intermittent storage of calcein in living cells. Normally, calcein does not pass cell membranes. An adsorption to organic linings therefore does not appear likely.

Due to the above-mentioned constraints, chamber formation of *Ammonia aomoriensis* could be assessed only by means of increase in test diameter. An examination of SEM images taken from 24 specimens of *A. aomoriensis* sampled in Flensburg Fjord, Kiel Fjord, and Eckernförde Bight (Schönfeld & Numberger 2007a,b, Polovodova & Schönfeld 2008, Nikulina et al. 2008, Polovodova et al. 2009, Schweizer et al. 2010) revealed an increase in diameter (average ± SD, n = 12) of 27 ± 16 µm per new chamber over the last 4 chambers. The height of the last 2 chambers, as seen from the spiral side, was 106 ± 23 µm. The increase in test diameter by adding new chambers did not covary with the overall diameter. Therefore we conclude that *A. aomoriensis* growth by 13 to 39 µm at a pCO_2 of 618 µatm corresponded to the addition of 1 or 2 new chambers during the 6 wk experimental period. In terms of chamber addition, active specimens of size fraction 150 to 250 µm added 2.3 chambers on average, and specimens of size fraction 250 to 350 µm added 1.6 new chambers. Inactive specimens added 1.0 new chamber on average (Table 3). An example of growth for one active individual of *A. aomoriensis* from size fraction 250 to 350 µm is presented in Fig. 7. During the incubation time, this individual grew by 33 µm. The growth took place in 2 increments of 16 µm, the first between Days 7 and 14, and the second between Days 14 and 21. On the basis of the aforementioned calculations, we concluded that this specimen had added 2 new chambers at a pCO_2 of 618 µatm.

Culture experiments assessing the growth of *Ammonia aomoriensis* from the western Baltic Sea have not been reported to date. The cultivation of *A. tepida* from San Antonio Bay, Texas, revealed a strong dependency of test growth on ambient temperature and salinity (Bradshaw, 1957). During the experimental period, we measured an average temperature of 11.7°C and salinity of 18.4 psu. On the basis of Bradshaw's (1957) data applied to our experimental settings, we estimated a growth rate of 0.06 chambers per day, causing us to expect an addition of 2 to 3 new chambers over 6 wk. In fact, our measurements indicated an addition of only 1 to 2 new chambers on average, which is slightly lower than could be estimated using Bradshaw's (1957) data, but it is nonetheless in general agreement with that estimate.

Dissard et al. (2009) reported that only half the specimens added new chambers during 6 wk of laboratory cultivation. Their average rates of 0.9 to 1.7 new chambers per individual fit well with our results. The culturing of the benthic foraminifer *Elphidium williamsoni* showed that they formed from 1 to 3 new chambers at a pH range of 7.6 to 8.3 at 15°C during an 8 wk experimental period (Allison et al. 2010).

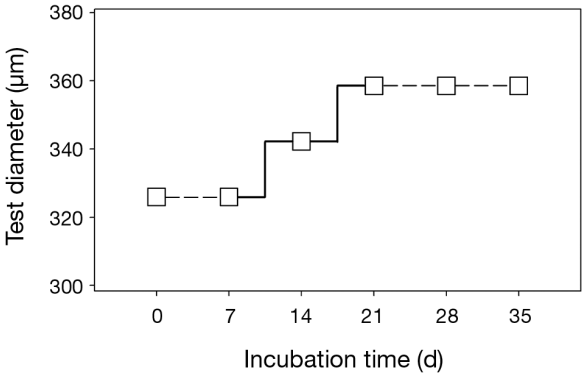

Fig. 7. *Ammonia aomoriensis.* Change in test diameter over time of a single active individual from size fraction 250–350 µm. Increment in diameter between Days 7 and 21 implies addition of 2 new chambers

During our experimental period, we observed that tests dissolved more readily at a high seawater pCO_2 than at a lower level. In our study the test diameter of *Ammonia aomoriensis* showed an increase with pCO_2 values up to 751 µatm. Above a critical pCO_2 level of 1829 µatm, however, dissolution features and a reduction of test diameter were observed. The inferred reduction of calcification might be a result of a presumably higher energetic cost to elevate the pH of intracellular vesicles where the first calcite crystals for the new chamber walls are precipitated (de Nooijer et al. 2008). This might explain the growth deceleration but not the size reduction observed at a pCO_2 at >1829 µatm. Alternatively, shell-wall thinning might be the result of dissolution under elevated pCO_2. SEM observations revealed the outer chamber walls to be 4 to 10 µm thick. Corrosion or even dissolution of the outer chamber walls would cause a reduction of the test diameter by 10 to 20 µm. The average chamber height is 106 µm. If the entire last whorl were to be dissolved, the shell loss would exceed by far the observed reduction of 23 to 49 µm in diameter. Therefore, it is reasonable to assume that the size reduction was due to a loss of outer shell wall and partial collapse of inner organic lining, which is in agreement with the SEM observations of dissolution features.

Dissolution features

Our results revealed a clear relationship between seawater pCO_2 and shell dissolution. The first dissolution features were recorded at a pCO_2 of 929 µatm and led to loss of the last-formed (thinner) chambers. At a

pCO_2 of 3130 µatm all chambers were destroyed by complete calcium carbonate dissolution, only the inner organic lining stayed intact.

Under elevated pCO_2 conditions, we observed different stages of dissolution. Decalcification started with loss of the external walls of the last chambers at a pCO_2 level of 929 µatm. The younger chambers decalcify first because their walls consist of a lower number of lamellae and therefore are thinner (Le Cadre et al. 2003). The next stage of dissolution is total decalcification of the outer walls and the inner organic lining, with cytoplasm becoming visible at a pCO_2 of 3130 µatm. For instance, the individual presented in Fig. 5D had a test diameter of 342 µm at the beginning, and after 6 wk incubation at pCO_2 of 3130 µatm the diameter was reduced to 32 µm.

The shell of foraminifers is composed of many calcitic layers — so called primary and secondary calcite — which cover the chambers (Erez 2003). The thickness of each layer depends on the number of chambers per whorl (Reiss 1957, Bentov & Erez 2005). The needles of the primary calcite, which forms the inner lamella outlining the new chamber, usually consist of a high-Mg calcite. The secondary calcitic layer, which covers the inner lamella as well as the entire existing shell, consists of low-Mg calcite (Reiss 1957, Erez 2003). Dissolution of the secondary calcite, which is deposited among the primary calcite needles, leads to test transparency (Le Cadre et al. 2003). We even observed a scabbing of the external walls of all chambers at a pCO_2 of 929 µatm (pH 7.7), with the primary calcite dissolving first, and needles of the secondary calcite beginning to thin (Fig. 6A). At a higher pCO_2 level (1829 µatm), primary calcite dissolved first and caused formation of lacunae among the needles of the first layer of secondary calcite and the inner organic lining. At the same time, the needles of the secondary calcite thinned at their base (Fig. 6B). Furthermore, the pore diameter expanded on the external walls and cracks were formed on the surface. At the highest pCO_2 treatment (3130 µatm), the primary calcite dissolved completely. In the first layer of secondary calcite from the inner side, the needles became generally thinner (Fig. 6D). The second layer of secondary calcite on the outer side corroded completely (Fig. 6D). In general, corroded tests became opaque. We conclude from our SEM observations that dissolution progressed both from the inner (cytoplasm) surface and the outer (seawater) surface. Acidified seawater probably diffuses through the pores from the external walls towards the inner organic lining. From the inner side the primary calcite corroded first. This is because this primary material consists of high-Mg calcite (Bentov & Erez 2005), which is less resistant to dissolution than the needles of secondary, low-Mg calcite.

Corroded walls have likewise been observed in living *Ammonia beccarii* from Isle de Yeu, France (Le Cadre et al. 2003). The specimens first retarded their pseudopodial network, then the test became opaque and the youngest chambers were destroyed. After 15 d, only the interlocular walls were preserved, and the inner organic layer covered the cytoplasm at the other parts of the test (Le Cadre et al. 2003).

The results of Le Cadre et al. (2003) demonstrate that *Ammonia beccarii* is able to rebuild its shells through recalcification when pH is increased following temporary exposure to low pH levels. In our laboratory experiment, however, *A. aomoriensis* was permanently exposed to low pH. According to our observations, *A. aomoriensis* exhibited no evidence of counteracting dissolution through rebuilding its shells during the incubation time of 6 wk.

Our laboratory experiment reproduced the dissolution phenomena observed in nature. Different stages of test dissolution of *Ammonia beccarii* were found in Gelting Bay, Flensburg Fjord. All observed *Ammonia* specimens were corroded and exhibited loss of the youngest chambers or the tests took on a star-like appearance with visible inner organic linings (Polovodova & Schönfeld 2008, Plate 3, Figs. 2–6). Similar dissolution features were observed in the following: *A. batavus* from Sandebukta, Oslo Fjord (Alve & Nagy 1986); *A. parkinsoniana, Elphidium excavatum* and *Palmerinalla palmerae* from Nueces Bay, Texas (Buzas-Stephens & Buzas 2005); tropical, intertidal benthic foraminifera from Cleveland Bay, North Queensland (Berkeley et al. 2008); and estuarine foraminifera from South Alligator River, Northern Territory, Australia (Wang & Chappell 2001).

The dissolution features observed in nature may have a variety of anthropogenic or natural causes (Le Cadre et al. 2003). Here we can only speculate that the lowering of pH in seawater of natural habitats is an important factor in test dissolution. Abrasion and predation, as well as early diagenesis, were previously considered as mechanisms that may act independently or amplify each other (Bradshaw 1957, Martin et al. 1995, Alve & Murray 1999, Moreno et al. 2007, Polovodova & Schönfeld 2008). As these processes can be ruled out under laboratory conditions, the shell loss of cultured foraminifers can only be interpreted in light of carbonate chemistry impacts on the calcification and dissolution process (Stubbles et al. 1996a,b).

Loss rate and mortality

We observed no significant differences of loss between the active and inactive specimens. At a pCO_2 of 3130 µatm, however, where significantly higher

losses were observed, the highest loss was seen in inactive specimens >200 µm, followed by small active specimens of the 150 to 250 µm size fraction. Subsequent treatment with Rose Bengal demonstrated that most of the specimens were devoid of cytoplasm at a pCO_2 of 3130 µatm. We observed that the test wall of *Ammonia aomoriensis* was completely destroyed at a pCO_2 of 3130 µatm and that only the inner organic lining was left. Therefore it is possible that the inner organic lining, which is much lighter than a calcitic test, may float easily or disappear. This may explain why we did not recover some of the specimens after 6 wk of incubation time. On the other hand, it is also possible that specimens were flushed away or escaped from the recruitment pits.

Loss and mortality rates revealed that inactive specimens or empty shells of foraminifera were affected first, followed by small active specimens of the 150 to 250 µm size fraction. This indicates that living cells may be able to counteract dissolution better than dead cells, at least up to a certain pCO_2 level. This emphasizes the potential for biological control, which is required to maintain inorganic tests and shells in an adverse abiotic environment.

Furthermore, we observed that the test walls of small specimens of the 150 to 250 µm size class sustained greater damage at high pCO_2 levels than did large specimens of the 250 to 350 µm size fraction. The surface:volume ratio of small tests is greater than that of large tests. Small specimens have a relatively greater surface, which is affected by external corrosion and may therefore respond more sensitively to undersaturated conditions. Another possible reason for greater damage sustained by small specimens is the thickness of the test walls of such specimens. In comparison to adult and large specimens, young and small specimens have thinner walls and fewer calcite lamellae. Therefore, the test walls of small specimens could be more easily destroyed. Our results indicate that the test walls of *Ammonia aomoriensis* cracked or dissolved at the high pCO_2 of 3130 µatm, first in inactive or dead specimens, then in small and finally in large specimens.

Ecological effect

Ammonia species are the most successful colonisers in near-coastal environments, and well-known opportunists, able to tolerate environmental stress (e.g. Almogi-Labin et al. 1995, Debenay et al. 1998, 2009). In the western Baltic Sea, *A. beccarii* was considered an invasive species, which arrived from the North Sea and finally colonised the area in the 20th century (Polovodova et al. 2009, Schweizer et al. 2010).

Field studies of Polovodova et al. (2009) showed fine porosity on the tests of living *Ammonia* from Flensburg Fjord (Stn PF16-25). In general, pores were seen to be joined in places, and tests showed signs of secondary calcification—e.g. regeneration scars. The observed porosity of *Ammonia* tests caused by dissolution may be explained by seasonal changes of the carbonate system under natural conditions (Table 1) and the ability of *Ammonia* to regenerate tests when conditions become less corrosive (Le Cadre et al. 2003). This is in contrast to the constantly elevated CO_2 levels simulated in our laboratory experiment.

While open ocean pCO_2 levels of 1829 (pH 7.4) and 3130 µatm (pH 7.2), as produced in this study, are not projected to develop due to ocean acidification in the near future, such conditions are already prevailing today in seasonally or permanently suboxic waters, including our sampling site in Flensburg Fjord. Because of the low buffering capacity of Baltic Sea water and the widespread seasonal undersaturation of portions of its bottom waters (Thomsen et al. 2010), the Baltic Sea is considered particularly vulnerable to acidification. Based on the results of this study, the resultant reduced calcification and shell dissolution of *A. aomoriensis* could lead to its disappearance from the Baltic Sea during the course of this century. This will also lead to changes in the community structure of benthic foraminifera (Watkins 1961, Schafer 1973, Ellison et al. 1986, Sharifi et al. 1991, Alve 1991, Yanko et al. 1998, Thomas et al. 2000, Debenay et al. 2001) and may induce shifts in the benthic ecosystem of the SW Baltic Sea.

CONCLUSIONS

Ammonia aomoriensis exhibited reduced calcification and increased test dissolution at elevated pCO_2 levels and lowered pH. Decalcification started with loss of the outer, thinner chambers at a pCO_2 of 929 µatm. Total decalcification, when chambers were destroyed and the inner organic lining became visible, began at a pCO_2 of 3130 µatm. Our observations indicate that dissolution of calcified structures progressed both from the inner (cytoplasm) surface and the outer (seawater) surface. Primary calcite is affected before secondary calcite. Observed loss and mortality rates suggest that living cells of *A. aomoriensis* are able to withstand and cope with dissolution up to a certain pCO_2 level. We have already achieved pCO_2 levels in the range of from 1829 (pH 7.4) to 3130 µatm (pH 7.2) during the seasonal cycle in shallow areas of Flensburg Fjord. With progressing CO_2-induced acidification this may eventually lead to conditions inducing significant changes in the composition of benthic foraminiferal

communities in our study area as well as in other regions experiencing naturally high bottom-water pCO_2 levels.

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