Proton pumping accompanies calcification in foraminifera

Takashi Toyofuku1,*, Miki Y. Matsuo2,*, Lennart Jan de Nooijer3,*, Yukiko Nagai1, Sachiko Kawada1, Kazuhiko Fujita4, Gert-Jan Reichart3,5, Hidetaka Nomaki6, Masashi Tsuchiya1, Hide Sakaguchi2 & Hiroshi Kitazato7

Ongoing ocean acidification is widely reported to reduce the ability of calcifying marine organisms to produce their shells and skeletons. Whereas increased dissolution due to acidification is a largely inorganic process, strong organismal control over biomineralization influences calcification and hence complicates predicting the response of marine calcifiers. Here we show that calcification is driven by rapid transformation of bicarbonate into carbonate inside the cytoplasm, achieved by active outward proton pumping. Moreover, this proton flux is maintained over a wide range of \( pCO_2 \) levels. We furthermore show that a V-type \( H^+ \) ATPase is responsible for the proton flux and thereby calcification. External transformation of bicarbonate into \( CO_2 \) due to the proton pumping implies that biomineralization does not rely on availability of carbonate ions, but total dissolved \( CO_2 \) may not reduce calcification, thereby potentially maintaining the current global marine carbonate production.

1 Department of Marine Biodiversity Research (B-DIVE), Japan Agency for Marine-Earth Science and Technology (JAMSTEC), Natsushima-cho 2-15, Yokosuka 237-0061, Japan. 2 Department of Mathematical Science and Advanced Technology (MAT), Yokohama Institute for Earth Sciences (YES), Japan Agency for Marine-Earth Science and Technology (JAMSTEC), 3173-25, Showa-machi, Kanazawa-ku, Yokohama-City, Kanagawa 236-0001, Japan. 3 Department of Ocean Systems, NIOZ-Royal Netherlands Institute for Sea Research and Utrecht University, Landsdiep 4, 1797 SZ ’t Horntje, The Netherlands. 4 Department of Physics and Earth Sciences, Faculty of Science and Tropical Biosphere Research Center, University of the Ryukyus, 1 Senbaru, Nishihara, Okinawa 903-0213, Japan. 5 Department of Earth Sciences - Geochemistry, Faculty of Geosciences, Utrecht University, P.O. Box 80.021, 3508 TA Utrecht, The Netherlands. 6 Department of Biogeochemistry, Japan Agency for Marine-Earth Science and Technology (JAMSTEC), Natsushima-cho 2-15, Yokosuka 237-0061, Japan. 7 Tokyo University of Marine Science and Technology, Konan 4-5-7, Minato-ku, Tokyo 108-8477, Japan. * These authors contributed equally to this work. Correspondence and requests for materials should be addressed to T.T. (email: toyofuku@jamstec.go.jp).
Marine calcification plays an important role in the global carbon cycle and it is estimated that up to 90% of all carbon currently buried in the seafloor results from biogenic calcium carbonate production\(^1,2\). On geological timescales, CaCO\(_3\) production and pCO\(_2\) are largely decoupled as alkalinity is added to the ocean from weathering. However, on time scales up to hundreds of years, calcification increases pCO\(_2\) as the lowered alkalinity shifts the inorganic carbon speciation towards CO\(_2\). Results from culturing experiments mimicking ocean acidification showed contrasting responses of calcification: calcification was reduced in some species, whereas others were not affected\(^3\). A large portion of open ocean calcium carbonate production, between 20 and 50%, derives from perforate foraminifera\(^4,5\). Despite its clear importance for the global carbon cycle, the physiological processes responsible for calcification in foraminifera are poorly understood. The key to understanding foraminiferal calcification centres on the relation between carbon speciation in seawater and preferential uptake of these chemical species (CO\(_2\), bicarbonate and/or carbonate ions)\(^3,6\).

Foraminifera build their tests by sequentially adding chambers. When foraminifera add a new chamber, the protoplasm does not contain sufficient building blocks/materials for calcifying an entirely new chamber. Limited availability of carbonate ions in seawater dictates that foraminifera may require unrealistic volumes of seawater to produce new calcium carbonate\(^6\). Hence, calcification by foraminifera has been hypothesized to involve intracellular storage of calcium ions and inorganic carbon\(^7\), pH manipulation\(^6,8,9\) and active calcium\(^10\) and/or magnesium pumping\(^11\). These results and the variety of postulated mechanisms for foraminiferal calcification\(^6,7,10–13\) make it challenging to reliably predict response to changes in marine inorganic carbon perturbations. Carbon and calcium uptake mechanisms and rates have been based on a combination of (scanning and transmission electronic) microscopy observations\(^14–16\), isotope labelling\(^17\), microelectrode measurement\(^9,18\) and analysis of the elemental and stable isotopic composition of foraminiferal calcite\(^11,12\). Recently, this has been complemented by applying fluorescent indicators visualizing ion fluxes inside actively calcifying specimens\(^6,8,19–21\). Imaging extracellular pH around benthic perforate foraminifera allows carbon speciation during calcification outside these foraminifera to be assessed. Although microelectrode analyses previously shows potential changes in carbon speciation outside the cytoplasm\(^9\), it remains to be quantified whether, and to what degree, different carbonate species contribute to calcification.

Here we show external pH change throughout the calcification of perforate foraminifera Ammonia sp., at a range of pCO\(_2\). Our results allow the calculation of proton fluxes and hence establish a quantitative calcification budget. Our physical model for calcification shows the dependence of foraminiferal biomineralization on the various inorganic carbon species present in seawater. We validate the importance of pH regulation on the foraminiferal calcification by application of a V-type H\(^+\) ATPase inhibitor, which plays a key role in aragonite production in scleractinian corals\(^22,23\).

**Results**

**External pH around foraminifera during chamber formation.** The first visualization of the extracellular spatial distribution of pH during chamber formation shows a strong decrease in external pH surrounding specimens of the benthic non-symbiotic foraminifer Ammonia sp. (Fig. 1, Table 1 and Supplementary Movie 1). This decrease in pH is modest at the start of chamber formation and intensifies over time in all five specimens studied, decreasing to a minimum value of \(\sim 6.9\) about 6 h after the start of calcification. The strongest pH decrease is observed closest to the newly forming chamber. It is noteworthy that minimum pH values are decreasing to 6.9 after 4 h and subsequently gradually increasing again 6 h after the onset of calcification. It is noteworthy that minimum pH values are found closest to the newly precipitated chamber (N). In addition, a zone of reduced pH encloses the complete shell, also where no new chamber is being produced. The gradient in pH, increasing with distance from the specimen, is mainly caused by protons diffusing away from the site where the new calcite is precipitated. Scale bars, 100 \(\mu\)m. The false-colour scale bar represents pH. The b/w foraminifer is superimposed on false-colour pH images.

**Figure 1 | Reduction in pH during foraminiferal calcification.** Representative images showing the time-resolved decrease in pH of seawater surrounding a calcifying specimen of Ammonia sp. over a period of 320 min. The pH values are imaged using dissolved HPTS and reported on the seawater scale. The incubated specimen shows (a) the two-dimensional variability in pH around the shell when building a new chamber and (b) the translated, spatially integrated change in pH versus distance from the foraminifer along the white dotted line shown in a. At the start of calcification, surrounding pH is still \(\sim 7.8\) outside the foraminifer, decreasing to 6.9 after 4 h and subsequently gradually increasing again 6 h after the onset of calcification. It is noteworthy that minimum pH values are found closest to the newly precipitated chamber (N).
between 6 and 12 h after calcification commences), external pH returned to ambient, pre-chamber formation values.

This decrease in external pH was observed over a wide range of pCO₂ (Table 1) and the reduction in pH compared with that of the ambient seawater was relatively constant over the experimental conditions. With a reduction in seawater pH by ~1 unit, the pH in the foraminiferal microenvironment also decreased by ~1 unit (Table 1). There was no clear relation between the foraminiferal size and the pH reduction, although specimens with the largest diameter were associated with the highest total proton flux (Table 1).

After addition of the V-type H⁺ ATPase inhibitor Bafilomycin A₁ at the onset of chamber formation, no clear external pH gradient develops, indicating a negligible proton flux. Occasionally, a very small decrease in external pH was observed during incubation with Bafilomycin (Table 1 and Supplementary Fig. 1). During these incubations, foraminifera produced very thin chamber walls, consisting mainly of the organic sheet produced at the beginning of new chamber formation (Supplementary Fig. 2).

**Discussion**

Combining time-resolved external pH recordings with two-dimensional pH gradient observations (lowest proximal to the newly formed chamber at 160 min; Fig. 1), allows calculating total proton flux \( Q_{\text{H}} \) from the site of calcification (SOC) to the specimen’s microenvironment (Fig. 2). The cumulative proton flux increase is relatively linear over time and results in a final cumulative proton flux. We found that the observed radial decrease in \([H^+]\) is well approximated by the second type of the modified spherical Bessel function, implying that the protons diffuse away from the foraminifer and that a proportion of them is consumed by carbonation during diffusion (for example, by the reaction with HCO₃⁻ to form CO₂ and H₂O). Proton flux originating from within the foraminifer is calculated by fitting the Bessel function and using Fick’s law (see ‘Modelling proton flux’ in Methods). The shape of the foraminifer is here considered spherical with a radius \( R = 100 \mu m \) and the proton flux is regarded homogenous over the complete specimen’s surface. Total proton flux thus integrates flux over the surface of the protective envelope (estimated to be 0.03 mm²; Fig. 1). For an average decrease in pH (0.5 at the surface of the specimen and 0.1 at a distance of 100 μm), an indicated specimen releases protons by an average flux \( Q_{\text{H}} = 0.014 \text{ mmol h}^{-1} \). The final cumulative proton flux (4–68 pmol; Table 1) is in the same order of magnitude as the total dissolved inorganic carbon (DIC) flux and 0.5 of the total amount of Ca²⁺ (2–34 pmol) necessary for the calcification of a new chamber. For a hemispherical chamber with a diameter of 20–50 μm, a wall thickness of 3 μm and a porosity of 25%, the required Ca²⁺ equals ~30–210 pmol. The similarity in fluxes may indicate that these processes are directly coupled, but may also be coincidental.

The observed decrease in pH outside the individual’s shell during calcification of benthic foraminifer Ammonia sp. implies that this foraminifer actively pump protons out of their protoplasm, with the flux independent of initial external pH (Fig. 3). Observation in the presence of the inhibitor Bafilomycin A₁ suggests that a V-type H⁺ ATPase is responsible for the proton transport (Supplementary Fig. 1). This is in line with earlier pH observations inside and outside calcifying foraminifera. The impact of decreased pH outside the foraminifer shifts inorganic carbon speciation as CO₃²⁻ is transformed into HCO₃⁻ and bicarbonate into CO₂ (Fig. 2). Within the SOC, elevated pH results in the opposite shift in speciation as HCO₃⁻ and CO₂ are transformed into CO₃²⁻ (Fig. 3). Hence, calcification is characterized by strong gradients in pH and pCO₂ between the SOC and the foraminiferal microenvironment (from 6.9 to 9 for pH and ~7,200 μatm to <20 μatm for pCO₂). Involvement of respired CO₂ may be responsible for part of the lowered pH. However, such a process is unlikely affected by the presence of Bafilomycin A₁, which prevented a clear pH decrease

---

**Table 1 | Summary of pH imaging observations during chamber formation.**

| No. | Total time of chamber formation (h:mm) | The lowest pH during an event (h:mm) | Shell diameter (μm) | Calculated total proton flux (pmol) | Ambient pH (seawater scale) | Estimated pCO₂ (μatm) |
|-----|--------------------------------------|-----------------------------------|-----------------|---------------------------------|--------------------------|-----------------------|
| 1   | 4:30                                 | 7.1 (2:25)                        | 141             | 6                               | 8.0                      | 460                   |
| 2   | 5:05                                 | 7.1 (3:25)                        | 216             | 17                              | 8.0                      | 460                   |
| 3   | 6:05                                 | 7.0 (3:50)                        | 323             | 68                              | 7.9                      | 610                   |
| 4   | 5:55                                 | 7.1 (2:55)                        | 166             | 6                               | 7.8                      | 790                   |
| 5   | 4:45                                 | 6.4 (2:30)                        | 228             | 14                              | 7.7                      | 1,030                 |
| 6   | 6:00                                 | 6.9 (2:52)                        | 260             | 58                              | 7.6                      | 1,320                 |
| 7   | 4:55                                 | 6.4 (1:45)                        | 268             | 15                              | 7.3                      | 2,160                 |
| 8   | 3:45                                 | 6.7 (2:45)                        | 243             | 4                               | 7.3                      | 2,760                 |
| 9   | 4:05                                 | 6.7 (1:00)                        | 203             | 6                               | 7.3                      | 2,760                 |
| 10  | 5:00                                 | 6.3 (1:15)                        | 186             | 5                               | 6.8                      | 9,010                 |

**With V type H⁺ ATPase inhibitor**

| No. | Total time of chamber formation (h:mm) | The lowest pH during an event (h:mm) | Shell diameter (μm) | Calculated total proton flux (pmol) | Ambient pH (seawater scale) | Estimated pCO₂ (μatm) |
|-----|--------------------------------------|-----------------------------------|-----------------|---------------------------------|--------------------------|-----------------------|
| 11  | 2:15                                 | 7.2 (1:20)                        | 231             | nd                              | 7.5                      | 1,560                 |
| 12  | 1:50                                 | 7.1 (1:05)                        | 256             | nd                              | 7.5                      | 1,560                 |
| 13  | 8:00                                 | 7.4 (8:00)                        | 308             | nd                              | 7.5                      | 1,560                 |

Reproducibility of pH value < 0.15 and total alkalinity of the solution is 2.330 ± 0.15 μmol kg⁻¹.
Foraminifer producing a new chamber. The reduction in pH is seen over the entire foraminifer (inset), suggesting of CO2 into the SOC (II). Once inside, the CO2 reacts to form CO3\(^{-2}\) outside the PE. The large gradient in pCO\(_2\) across the PE results in diffusion of CO\(_2\) into the SOC (III). Once inside, the CO2 reacts to form CO3\(^{-2}\) due to the high pH (IV) sustaining CaCO3 precipitation by reacting with the Ca\(^{2+}\). The reduction in pH is seen over the entire foraminifer (inset), suggesting that this model applies to the complete surface of the shell of a foraminifer producing a new chamber.

During calcification of a new calcitic layer (CL) on a primary organic sheet (POS), the protective envelope (PE) separates the growing calcite surface from the surrounding seawater. The chemical composition at the SOC, created by the PE, is characterized by active, outward proton pumping (I). The reduced pH in the foraminiferal microenvironment shifts the inorganic carbon speciation (II), thereby increasing pCO\(_2\) directly outside the PE. The large gradient in pCO\(_2\) across the PE results in diffusion of CO\(_2\) into the SOC (III). Once inside, the CO2 reacts to form CO3\(^{-2}\) due to the high pH (IV) sustaining CaCO3 precipitation by reacting with the Ca\(^{2+}\). The reduction in pH is seen over the entire foraminifer (inset), suggesting that this model applies to the complete surface of the shell of a foraminifer producing a new chamber.

As CO\(_2\) diffuses easily across cell membranes compared to HCO\(_3^-\), the large pCO\(_2\) gradient results in a flux of carbon dioxide into the foraminifer (Fig. 3). The high pH at the SOC locally increases saturation state and hence promotes calcification (Fig. 3). Inside the specimen, excess protons from the conversion of CO\(_2\) into (bi)carbonate help sustain CaCO\(_3\) production by reacting with the Ca\(^{2+}\) and the continued proton flux outside of the foraminifer (Fig. 3).

Modelling proton pumping to mimic the observed pH gradient outside the specimen over time (Fig. 1) implies that more than half of the protons are consumed by the reaction with bicarbonate. Therefore, the calculated increase in pCO\(_2\) converts between 25 and 50% of all DIC into carbon dioxide directly outside the foraminifer. The exact value converted depends on the appropriate dissociation constant for the conversion between CO2 and HCO3\(^-\), and on the exact pH of the foraminifer’s microenvironment. The rate at which this CO2 is taken up by the foraminifer depends on the thickness of the pseudopodial envelope across which the CO2 diffuses and the constant rates for the reactions of the inorganic carbon species at the SOC (Fig. 3). The hydration of CO2 to form bicarbonate and a proton is relatively slow and could therefore limit calcification rates. The slow kinetics of this reaction may however be ‘bypassed’ by CO2 reacting with OH\(^-\) at the SOC. Alternatively, the conversion rate may be increased by the presence of specialized enzymes like carbonic anhydrase, which are known/suggested to be involved in the calcification of other marine calcifiers including corals\(^{23}\), coccolithophores\(^{25}\) and bivalves\(^{26}\). Although not relevant for the fluxes calculated here, ultimately a more precise characterization of the chemical composition at the SOC is necessary to show the relative contribution of these pathways to the overall conversion of CO\(_2\) into carbonate.

Culture studies using planktonic foraminifera show that the high pH (IV) sustaining CaCO3 precipitation by reacting with the Ca\(^{2+}\) and the continued proton flux outside of the foraminifer (Fig. 3). The slow kinetics of this reaction may however be ‘bypassed’ by CO2 reacting with OH\(^-\) at the SOC. Alternatively, the conversion rate may be increased by the presence of specialized enzymes like carbonic anhydrase, which are known/suggested to be involved in the calcification of other...
by such variations in ambient pH, as the foraminifer-induced pH changes exceed those occurring naturally.

Methods

Specimens. Culture experiments and microscope observations were performed at the Japan Agency for Marine-Earth and Technology (JAMSTEC) laboratory, Yokosuka, Japan. The living specimens were collected from brackish water salt marsh sediments of Hiragata-bay, Natsushima-cho Yokosuka (33.3226°N, 139.6347°E). Ammonia sp. was used for the experiments, a benthic, hyaline, cosmopolitan species. Living specimens were isolated and cleaned from excess sediment and debris, transferred to filtered (0.2 μm) seawater and placed in a Petri dish. The dishes were maintained at 20°C in filtered seawater with a pH of ~7.9 and a pCO₂ of ~550 μatm. Once a week, the seawater was replaced and live algae (Dunaliella tertiolecta) were added as food.

Ambient pH distributions were visualized around foraminiferal specimens that were starting to form a new chamber. We identified specimens close to forming a new chamber by the presence of excess fluffy material (for example, clastics and algae), forming a protective cyst, surrounding a fan-like pseudopodal network in the shape of a new chamber. At that moment, an organic membrane is expanding on the pseudopodal network, delineating the shape of the soon-to-be-built chamber. This organic membrane, also known as the primary organic sheet, serves as a template on which the first calcite of the new chamber precipitates. Specimens are cultured within 35 mm glass base dishes (3910-035, Ibuki glass).

Observation settings. For ambient pH imaging, pH indicator HPTS (pyrane 8-Hydroxypropene-1,3,6-trisulfonyl acid trisodium salt, H13259, Sigma-Aldrich) was dissolved to a final concentration of 20 mM. This concentration of HPTS is known to be harmless to foraminiferal behavior and does not noticeably impair their calcification processes. Total alkalinity of the solution is determined by method 40. The observations were carried out with ten individuals under room temperature (~23°C). The individuals were then observed under an inverted fluorescent microscope (Zeiss Axios Observer Z1, Germany).

Three individuals were additionally incubated with Bafilomycin A₁, a V-type ATPase inhibitor (BVT-0252, BioVitocia). These incubations were done to investigate the influence of H⁺ ATPases on calcification (see similar approach in scleractinian corals). Ammonia sp. was dissolved to a final concentration of 1 μM in seawater with 20 μM HPTS. The specimens were placed in the solution only during chamber formation. All three specimens were observed trying to form a new chamber in the presence of Bafilomycin A₁.

Optical settings. Fluorescent filter cubes were used to detect pH signals from HPTS (510 exc–550–560 nm, 540 emission) and HPTS (510 exc–550–560 nm). Time-lapse images were captured every 5 min by a digital camera attached to the microscope using a standard software package (Axiovision, Version 4.6). Grey scale images representing different emission wave length intensities were exported as TIFF files. Subsequently, ratiometric pH images were calculated by dividing 540/510 nm for each pixel, using a custom calibration curve (Supp. Fig. 3). The pCO₂ of each medium was estimated using the CO₂SYS software package after determining pH of the media ratiometrically and using total alkalinity.

Observation management. The pH of HPTS solution is manipulated by CO₂ bubbling just before the experimental incubation. The pH of the solution was continuously monitored by a pH meter (Thermo Scientific Orion 5-star Plus) equipped with a glass electrode (Thermo Scientific, PpHect ROSS Micro Combination pH electrode 8220BNWP) to ensure the appropriate amount of CO₂ was added. The pH values are indicated with the seawater scales.

The natural medium was replaced by seawater containing HPTS solution three times by removal of the seawater with a Pasteur pipette and subsequent addition of the HPTS-containing seawater. The pipetting was done very gently to avoid disturbance of any foraminiferal activities or to minimize gas exchange. The water's surface was covered by a cover glass to prevent gas exchange between water and air during observation. The pH was increased until the equivalent state reached the laboratory's atmospheric pCO₂ if the cover had not been used.

Modelling proton flux. First, we considered a model of proton release for a foraminifer. For simplicity, we assume that the foraminifer is spherical with radius R and it is covered by a thin protective envelope. It is assumed that protons are released from the protective envelope and outside the foraminifer and protons diffuse, and at the same time are consumed due to the carbonation reaction: [H⁺] + [CO₃²⁻] → [HCO₃⁻]. The reverse reaction is assumed not to occur, which is realistic due to the relatively low pH outside the specimen. With these assumptions, the proton concentration outside the foraminiferal cell can be calculated using a diffusion equation with added consumption:

$$\frac{\partial}{\partial r}[H^+] = -D_H \frac{\partial^2 [H^+]}{\partial r^2} - \mu [H^+] - [H^+]_{\infty},$$

where $D_H$ is the diffusion constant of proton in seawater and $\mu$ is the constant rate of the carbonation reaction.

We solve this equation under the boundary conditions: $[H^+] = [H^+]_{\infty}$ at $r \to \infty$ and $[H^+] = [H^+]_0$ at $r \to R$, where $[H^+]_0$ is the equilibrium concentration of protons in natural seawater and the value of $[H^+]_0$ is controlled by the foraminifer, depending on its developmental stage. When the foraminifer begins building a new chamber, $[H^+]_0$ becomes larger than $[H^+]_{\infty}$. After some time, equilibrium has established and the spatial distribution of proton obeys the steady solution of equation (1) described by

$$[H^+] = K_{H,z}(a)/(\gamma r^m) + [H^+]_{\infty},$$

where $K_{H,z}$ is the second type of the modified Bessel function $K_0$ with $a = 1/2$ and $m = \sqrt{2N}/r$. The local radial flux of proton on the protective envelope is calculated using Fick's law,

$$J_k = D_H \left( \frac{\partial [H^+]}{\partial r} \right)_{r=R}$$

When the shape of the foraminifer is spherically symmetric, the total flux is calculated by

$$Q_{H} = 4\pi R^2 J_k.$$

Thus, the total flux $Q_{H}$ is determined by equations (2–4). We accordingly calculated the total proton flux of a foraminiferal specimen from its pH image. The nonlinear, least square fitting of the radial distribution of protons by equation (2) determines the values of coefficients $a$, $m$, and $[H^+]_{\infty}$.

Data availability. The data in this study are available from the corresponding author on the reasonable request.

References

1. Feely, R. A. et al. Impact of anthropogenic CO₂ on the CaCO₃ system in the oceans. *Science* 305, 362–366 (2004).

2. Sarmento, J. L. & Gruber, N. Ocean Biogeochemical Dynamics 528 (Princeton Univ. Press, 2006).

3. Doney, S. C., Fabry, V. J., Feely, R. A. & Kleypas, J. A. Ocean acidification: the other CO₂ problem. *Annu. Rev. Mar. Sci.* 1, 169–192 (2009).

4. Langer, M. R. Assessing the contribution of foraminiferan protists to global ocean carbonate production. *J. Eukaryotic Microbiol.* 55, 163–169 (2008).

5. Schiebel, R. Planktic foraminiferal sedimentation and the marine calcite budget. *Global Biogeochem. Cycles* 16, 3–13 (2002).

6. De Nooijer, L. J., Spero, H. J., Erez, J., Bijma, J. & Reichart, G. J. Biominalerization in perforate foraminifera. *Earth Sci. Rev.* 135, 48–58 (2014).

7. Erez, J. The source of ions for biominalerization in foraminifera and their implications for paleoceanographic proxies. *Rev. Mineral. Geochem.* 54, 115–149 (2003).

8. Béjar, S., Brownlee, C. & Erez, J. The role of seawater endocytosis in the biominalerization process in calcareous foraminifera. *Proc. Natl Acad. Sci. USA* 106, 21500–21504 (2009).

9. Glas, M. S., Langer, G. & Keul, N. Calcification acidifies the microenvironment of a benthic foraminifer (Ammonia sp.). *J. Exp. Mar. Biol. Ecol.* 424–425, 53–58 (2012).

10. Nehrke, G. et al. A new model for biominalerization and trace-element signatures of foraminifera tests. *Biogeosciences* 10, 6759–6767 (2013).

11. Bentov, S. & Erez, J. Impact of biominalerization processes on the Mg content of foraminiferal shells: a biological perspective. *Geochim. Geophys. Geosyst.* 7, Q01P08 (2006).

12. Elderfield, H., Bertram, C. J. & Erez, J. A biominalerization model for the incorporation of trace elements into foraminiferal calcium carbonate. *Earth Planet. Sci. Lett.* 142, 409–423 (1996).

13. De Nooijer, L. J., Langer, G., Nehrke, G. & Bijma, J. Physiological controls on seawater uptake and calcification in the benthic foraminifer *Ammonia tepida*. *Biogeosciences* 6, 2669–2673 (2009).

14. Banner, F. T., Sheehan, R. & Williams, E. The organic skeletons of rotalian foraminifera: a review. *J. Foram. Res.* 33, 30–42 (1973).

15. Bé, A. W. H., Hemleben, C., Anderson, O. R. & Spindler, M. Chamber formation in planktonic foraminifera. *Micropaleontol.* 25, 294–307 (1979).

16. Hemleben, C., Erson, O. R., Berthold, W. & Spindler, M. in *Biominalerization in Lower Plants and Animals* (eds Leadbeater, B. S. & C. R. & Riding, R.) 237–249 (Clarendon Press, 1986).

17. Ter Kuijle, B. H. & Erez, J. Carbon budgets for two species of benthonic symbiont-bearing foraminifera. *Bioll. Bull.* 180, 489–495 (1991).
22. Barott, K. L., Venn, A. A., Perez, S. O., Tambutte, S. & Tresguerres, M. and Moya, A.

20. De Nooijer, L. J., Toyofuku, T., Oguri, K., Nomaki, H. & Kitazato, H. Intracellular pH distribution in foraminifera determined by the fluorescent probe HPTS. Limnol. Oceanogr. Methods 6, 610–618 (2008).

21. De Nooijer, L. J., Toyofuku, T. & Kitazato, H. Foraminifera promote calcification by elevating their intracellular pH. Proc. Natl Acad. Sci. USA 106, 15374–15378 (2009).

25. Rickaby, R. E. M., Henderiks, J. & Young, J. N. Perturbing phytoplankton: response and isotopic fractionation with changing carbonate chemistry in two cocolithophorid species. Clim. Past 6, 771–785 (2010).

26. McConnaughey, T. A. & Gillikin, D. P. Carbon isotopes in mollusk shell carbonates. Geochim. Cosmochim. Acta 65, 311–122 (2001).

27. Spero, H. J., Bijma, J., Lea, D. W. & Bemis, B. E. Effect of seawater carbonate concentration on foraminiferal carbon and oxygen isotopes. Nature 390, 497–500 (1997).

28. Zhang, J., Quay, P. D. & Wilbur, D. O. Carbon isotope fractionation during gas-exchange and dissolution of CO2. Geochim. Cosmochim. Acta 59, 107–114 (1995).

29. McCorkle, D. C., Corliss, B. H. & Farnham, C. A. Vertical distributions and isotopic composition of live (stained) benthic foraminifera from the North Carolina and Carolina continental margins. Deep Sea Res. I 44, 983–1024 (1997).

30. Bijnia, J., Hemleben, C., Huber, B. T., Erlenkeuser, H. & Kroon, D. Experimental determination of the ontogenetic stable isotope variability in two morphotypes of Globigerinella siphonifera (d’Orbigny). Mar. Micropaleontol. 35, 141–160 (1998).

31. Zeebe, R. E. & Fröhlich, A. Comparison of two potential strategies of planktonic foraminifera for building MgCO3: H+ exchange or biogenic MgCO3 precipitation? Geochim. Cosmochim. Acta 66, 1159–1169 (2002).

32. Fujita, K. et al. Effects of ocean acidification on calcification of symbiont-bearing reef foraminifers. Biogeosciences 8, 2089–2098 (2011).

33. Hikami, M. et al. Contrasting calcification responses to ocean acidification between two reef foraminifers harboring different algal symbionts. Geophys. Res. Lett. 38, L19601 (2011).

34. Keul, N., Langer, G., De Nooijer, L. J. & Bijnia, J. Effect of ocean acidification on the benthic foraminifera Ammonia sp. is caused by a decrease in carbonate ion concentration. Biogeosciences 10, 6185–6198 (2013).

35. Flako-Zaritsky, S., Almogi-Labin, A., Schulman, B., Rosenfeld, A. & Benjamini, C. The environmental setting and microfauna of the oligohaline Timnah pond, Israel: the last remnant of the Kabara swamps. Mar. Micropaleontol. 80, 74–88 (2011).

36. Caldeira, K. & Wickett, M. E. Oceanography: anthropogenic carbon and ocean pH. Nature 425, 365–368 (2003).

37. Ries, J. B. A physicochemical framework for interpreting the biological calcification response to CO2-induced ocean acidification. Geochim. Cosmochim. Acta 75, 4053–4064 (2011).

38. Murray, J. W. Ecology and Applications of Benthic Foraminifera (Cambridge Univ. Press, 2006).

39. Stahl, H. et al. Time-resolved pH imaging in marine sediments with a luminescent planar optode. Limnol. Oceanogr. Methods 4, 336–345 (2006).

40. Cullberson, C., Pytkowicz, R. M. & Hawley, J. E. Seawater alkalinity determination by the pH method. J. Mar. Res. 28, 15–21 (1970).

41. Dickson, A. G. & Goyet, C. Handbook of Methods for the Analysis of the Various Parameters of the Carbon Dioxide System in Sea Water. (Ver. 2) Department of Energy, ORNL/CDIC-74 (Oak Ridge, Tenn. 1994).

42. Bowman, E. J., Siebers, A. & Altenendorf, K. Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. Proc. Natl Acad. Sci. USA 85, 7972–7976 (1988).

43. Pierrot, D., Lewis, E. & Wallace, D. W. R. MS Excel Program Developed for CO2 System Calculations (Oak Ridge National Laboratory 1998).

Acknowledgements

We thank Mr Y. Tsuchiya (JAMSTEC) for video editing. We also thank Dr B. Mamo (The University of Hong Kong) for helpful discussions. This work was supported by JSPS KAKENHI Grant Numbers 22640427 (T.T.) and 25247085 (H.K.). This work was carried out under the programme of the Netherlands Earth System Science Center (NESSC).

Author contributions

Scientific conception and experimental design: T.T., H.K. and H.S. Data acquisition and analysis: T.T., M.Y.M., L.J.d.N., Y.N. and S.K. Collected and processed data: T.T., M.Y.M. and L.J.d.N. Data interpretation: T.T., M.Y.M., L.J.d.N., K.F., G.-J.R., H.N., M.T. Wrote paper: T.T., M.Y.M., L.J.d.N. and G.-J.R. All authors discussed the results and commented on and revised the manuscript.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/nc

Competing financial interests: The authors declare no competing financial interests.

Reprints and permission information is available online at http://npg.nature.com/npqapeutics/permissions/

How to cite this article: Toyofuku, T. et al. Proton pumping accompanies calcification in foraminifera. Nat. Commun. 8, 14145 doi: 10.1038/ncomms14145 (2017).

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.