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EDITED BY

Jin-e Liu,
Nanjing Normal University, China

REVIEWED BY

Veronica Rossi,
University of Bologna, Italy
Yvonne Milker,
University of Hamburg, Germany

*CORRESPONDENCE

Ying Zhang
✉ yingzhang@uri.edu

RECEIVED 04 February 2025

ACCEPTED 30 May 2025

PUBLISHED 10 July 2025

CITATION

Powers C, Paz A, Zyck A, Harri K, Geraci M,
Bernhard JM and Zhang Y (2025)
Morphological responses of a temperate
intertidal foraminifer, *Haynesina* sp., to coastal
acidification.
Front. Microbiol. 16:1570629.
doi: 10.3389/fmicb.2025.1570629

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Morphological responses of a temperate intertidal foraminifer, *Haynesina* sp., to coastal acidification

Christopher Powers^{1,2}, Alberto Paz¹, Amaelia Zyck²,
Kaylee Harri¹, Madison Geraci^{1,2}, Joan M. Bernhard³ and
Ying Zhang^{1*}

¹Department of Cell and Molecular Biology, College of the Environment and Life Sciences, University of Rhode Island, Kingston, RI, United States, ²Biological and Environmental Sciences Graduate Program, University of Rhode Island, Kingston, RI, United States, ³Department of Geology and Geophysics, Woods Hole Oceanographic Institution, Woods Hole, MA, United States

Coastal acidification could have widespread impact on marine organisms, affecting the ability of calcifying organisms to build shells and skeletons through calcium carbonate precipitation. As an abundant group of calcifying organisms, some protists within the phylum Foraminifera demonstrate potential success under elevated partial pressure of carbon dioxide ($p\text{CO}_2$) due to their ability to modulate intracellular pH. However, little is known about their responses under more extreme acidification conditions that are already seen in certain coastal environments. Here we exposed specimens of *Haynesina* sp., which belongs to a genus that is prevalent in temperate intertidal salt marshes, to moderate ($p\text{CO}_2 = 2386.05 \pm 97.14 \mu\text{atm}$) and high acidification ($p\text{CO}_2 = 4797.64 \pm 157.82 \mu\text{atm}$) conditions through the duration of 28 days. We demonstrate that although this species is capable of withstanding moderate levels of coastal acidification with little impact on overall test thickness, it can experience precipitation deficiency and even dissolution of the calcareous test under highly elevated $p\text{CO}_2$. Interestingly, such a deficit was primarily seen among live foraminifera, as compared to dead specimens, throughout the four-week experiment. This study suggests that a combination of environmental stress and the physiological process of test formation (i.e., calcite precipitation) could induce thinning of the test surface. Therefore, with the acceleration of coastal acidification due to anthropogenic production of CO_2 , benthic foraminifera and other calcifying organisms among coastal ecosystems could reach a tipping point that leads to thinning and dissolution of their calcareous tests, which in turn, will impair their ecological function as a carbon sink.

KEYWORDS

foraminifera, ocean acidification, *Haynesina*, $p\text{CO}_2$, calcification

Introduction

Increasing anthropogenic production of CO_2 into the atmosphere has resulted in rising ocean temperatures, causing increases in sea levels and changes in the ocean's chemistry (Stocker et al., 2014). As excess CO_2 dissolves in seawater, the concentration of hydrogen ions increases, leading to acidification and reduced carbonate ion concentration (Leung et al., 2022; Guinotte and Fabry, 2008; Doney et al., 2009). Anthropogenic-driven acidification has resulted in decreasing pH across the world's oceans, with drops of 0.1 to 0.3 pH units possible in open ocean waters within the next 100 years (Stocker et al., 2014). These changes are exacerbated in coastal areas, examples of which include Narragansett Bay (RI, United States) and Long

Island Sound (NY-CT-RI, United States), where low pH (<7.2), high $p\text{CO}_2$ (>2,500 μatm), and aragonite undersaturation ($\Omega_{\text{aragonite}} < 1$) have been observed periodically in bottom waters (Wallace et al., 2014).

Increased $p\text{CO}_2$ may have dire impacts for organisms in the ocean, such as those that produce their own shells, tests, or skeletons by depositing calcium carbonate minerals (i.e., calcite and aragonite). As $p\text{CO}_2$ of seawater increases, the aragonite saturation ($\Omega_{\text{aragonite}}$) and calcite saturation (Ω_{calcite}) state of seawater decreases, undermining the shell formation of marine organisms (Stocker et al., 2014; Figuerola et al., 2021; Andersson et al., 2008). However, the impact of ocean acidification on individual calcifying taxa remains difficult to generalize due to the confounding effects of elevated $p\text{CO}_2$ and organisms' own acclimatory responses (Leung et al., 2022; Melzner et al., 2020). Such phenomena call for more studies on specific taxa to gain a more complete picture of their responses to acidification.

The Foraminifera is a phylum of unicellular microeukaryotes prevalent in marine water columns and sedimentary ecosystems, from shallow and intertidal zones to deep water depths. Many foraminifera produce calcareous tests through calcite precipitation (Figuerola et al., 2021; Todd and Blackmon, 1956). Foraminifera account for an estimated 25% of calcium carbonate deposition across the world's oceans, attesting to their important roles in carbon and mineral cycling (Langer, 2008). The test morphology within certain foraminifera genera varies depending on their reproductive stage (Hottinger, 2000; Lei et al., 2017; Gudina and Levtschuk, 1989; Nigam, 1986; Nigam and Rao, 1987; Alve and Goldstein, 2003; Goldstein, 1999). The sexually reproducing microspheric foraminifera are characterized by a relatively small proloculus (i.e., the first chamber formed during growth) due to cytoplasmic requirements of progeny produced through gametogenesis and fertilization (Hottinger, 2000). In contrast, the asexually reproducing megalospheric foraminifera have a relatively large proloculus and inherit substantial volumes of the parental cytoplasm, including potential symbionts (Hottinger, 2000). Additionally, the microspheric foraminifera tend to form more chambers than their megalospheric counterparts while exhibiting heteromorphic test structure (Lei et al., 2017; Gudina and Levtschuk, 1989).

Despite abundance of calcifying foraminifera in temperate coastal systems, most acidification studies of foraminifera focused on species from reef-associated systems or the open ocean (Supplementary Table S1). A few studies examine the responses of individual foraminifera to coastal acidification (Khanna et al., 2013; Prazeres et al., 2015; McIntyre-Wressnig et al., 2011; Schmidt et al., 2014), but more extreme conditions related to what is seen in coastal habitats (e.g., $p\text{CO}_2 > 2,500 \mu\text{atm}$) are rarely considered. Common strategies for measuring foraminifera responses to acidification involve tracking chamber formation rates and surface morphological changes, with prior studies demonstrating variable influences of acidification on different species of foraminifera (Khanna et al., 2013; Prazeres et al., 2015; Schmidt et al., 2014; Keul et al., 2013; Haynert et al., 2014; Kuroyanagi et al., 2021; Manno et al., 2012; Kuroyanagi et al., 2009; Fujita et al., 2011; McIntyre-Wressnig et al., 2013; Raymond et al., 2013; Iwasaki et al., 2019; Sinutok et al., 2011; Bernhard et al., 2021). The direct measurement of test thickness, however, has not been systematically applied to study the acidification responses in calcareous foraminifera.

In this study, laboratory treatments were conducted to examine the response of *Haynesina* sp., a benthic foraminifer identified from

coastal sediments in Rhode Island, United States, to moderate and high acidification conditions. Thicknesses of foraminifera tests were mapped using X-ray tomography, enabling systematic comparisons throughout individual test chambers. The treatments were applied to both live and dead foraminifera, which provides an opportunity for examining the biotic and abiotic responses of foraminifera and their calcareous remains to coastal acidification. We hypothesized that acidification conditions would result in detectable thinning or morphological defects of foraminiferal tests at the end of the experimental period.

Results

Acidification challenges

Specimens were obtained from a mudflat associated with the Quonochontaug Salt Marsh (41°20'12.6"N, 71°43'15.9"W) on June 19, 2023 and September 26, 2023 for two replicate pH manipulation experiments (Table 1). Each replicate trial was performed over a period of 28 days with seawater chemistry closely monitored through three distinct $p\text{CO}_2$ regimes: no $p\text{CO}_2$ manipulation (431.18+/-18.90 μatm), moderately elevated $p\text{CO}_2$ (2386.05+/-97.14 μatm), and highly elevated $p\text{CO}_2$ (4797.64+/-157.82 μatm) (Figure 1A; Supplementary Table S2). Additionally, untreated specimens were obtained from sediment samples and used as additional controls (Materials and Methods). Measurements of the calcite saturation state indicated supersaturation ($\Omega_{\text{calcite}} > 1$) under both non-elevated (Tank 3) and moderately elevated (Tank 2) $p\text{CO}_2$ treatments. However, calcite undersaturation ($\Omega_{\text{calcite}} < 1$) was observed under the highly elevated (Tank 1) $p\text{CO}_2$ treatment (Table 1).

Microscopy and three-dimensional test reconstruction

Three-dimensional (3D) reconstruction of foraminifera tests was achieved with a voxel size of 0.57 μm (resolution around 1 μm) using microCT scanning (Figure 1B). Individual chambers were extracted during image processing following the 3D reconstruction, and the thicknesses of exteriorly facing test areas were measured (Figure 1C, Materials and Methods). From each replicate trial, we initially collected at least six live and six dead specimens from each treatment tank, and at least eight specimens as untreated controls. Some tests were lost during handling, and some others were damaged when mounted for microCT scanning due to their delicate nature. These structurally damaged tests were found among all treatment conditions and were not included in further analyses. In total, 76 specimens (28 live treated, 33 dead treated, and 15 untreated) were included in the final collection of the microCT scanning data (Supplementary Table S3).

Assignment of test morphology

Foraminifera specimens collected from each treatment were classified into two distinct groups, microspheric and megalospheric, based on their heteromorphic test geometry (Materials and Methods).

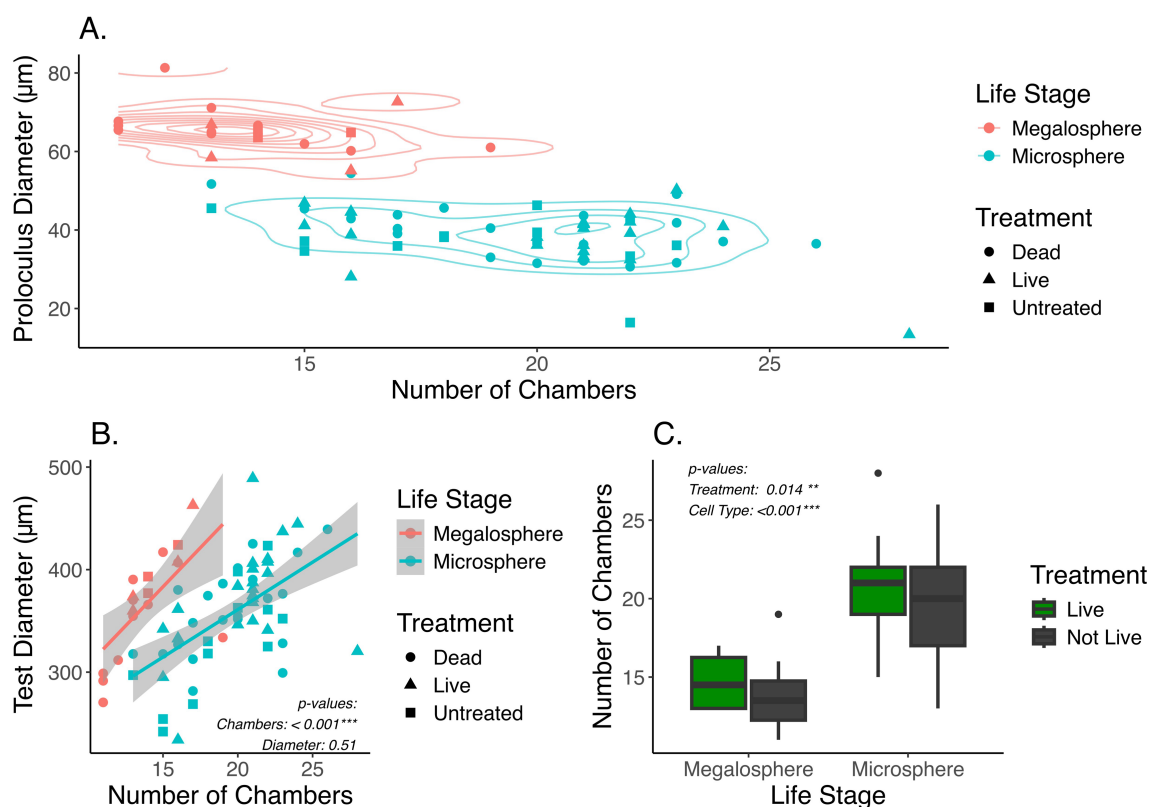


FIGURE 2

(A) Plot showing the number of chambers against the proloculus diameter. Each point represents a foraminifer. Color represents the assignment of two life stages, microsphere or megalosphere, based on the diameter of proloculus. Symbol shape represents different treatment groups, including untreated. (B) Comparison of the number of chambers and the test diameter between two life stages. *p*-values are based on one-way ANOVA accounting for different life stages (Materials and Methods). (C) Box and whisker plot showing distribution of the number of chambers in live and not-live foraminifera between the two life stages. The “not-live” specimens include both untreated and dead treated samples. *p*-values are based on two-way ANOVA accounting for life stages and live vs. not-live treatment groups (Materials and Methods).

variability in the number of chambers among individual foraminifera.

Variation of test thicknesses across different treatments

The test morphology was not assigned until after the experimental period due to challenges in keeping foraminifera alive following microCT scanning, which involves bleaching to remove soft tissues and the exposure to high X-rays through extended scanning period during imaging (Materials and Methods). According to our post-experimental assignment of test morphology, an uneven number of megalospheric and microspheric specimens were included in the different experimental treatments. Due to the sparsity of megalospheric samples in multiple treatment conditions (Supplementary Table S3), statistical comparison of test thicknesses across different treatment groups (e.g., treated versus untreated, different $p\text{CO}_2$ treatment conditions, or live versus dead treatments) were performed only with the microspheric specimens.

Comparisons of test thicknesses indicated substantial variations among individual foraminifera. The effect size (η^2) related to the

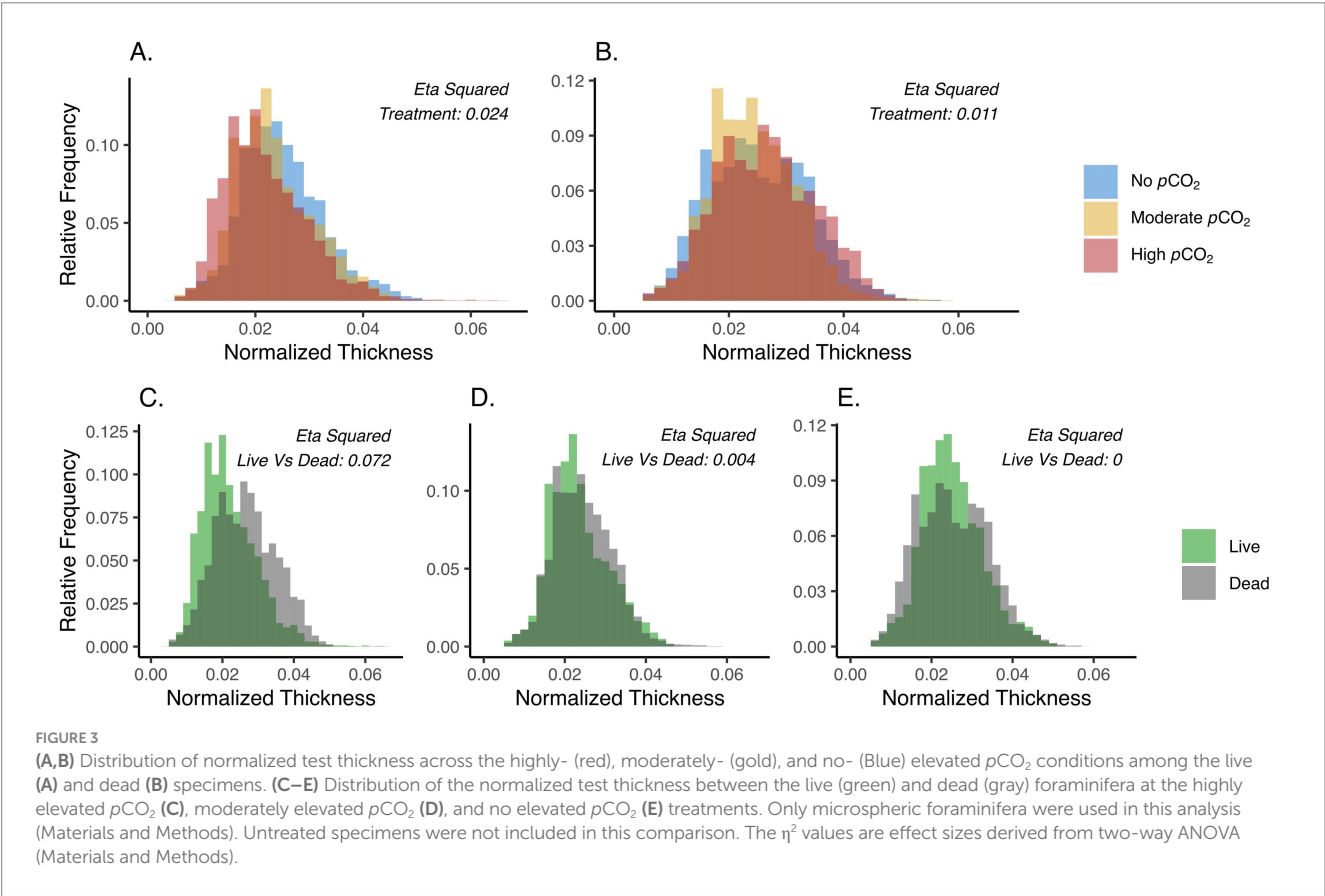
individual variance in two-way ANOVA analyses was around 0.283–0.365, which is 1–2 orders of magnitude higher than what was seen in the effect of experimental treatments (Table 2). Although a relatively small effect was seen in the factor that compared different treatments to the no-treatment control, they revealed variable responses. The non-elevated and moderately elevated $p\text{CO}_2$ treatments had negligible effect sizes ($\eta^2 \leq 0.01$) compared to the untreated control. In contrast, specimens in the highly elevated $p\text{CO}_2$ treatments had thinner tests compared to the untreated control, with effect sizes of 0.023 and 0.014, respectively, for the live and dead foraminifera (Table 2).

Significant differences in test thicknesses were observed for both live and dead specimens across the different $p\text{CO}_2$ treatments, where a slightly higher effect size was observed among the live ($\eta^2 = 0.024$) than the dead treatments ($\eta^2 = 0.011$) (Figures 3A,B). Specifically, thinner tests were observed in the live cell treatments under highly and moderately elevated $p\text{CO}_2$ compared to the non-elevated $p\text{CO}_2$ (Figure 3A). Differential responses between live and dead treatments were also observed in the distribution of test thicknesses. The largest effect was seen in the highly elevated $p\text{CO}_2$ treatment ($\eta^2 = 0.072$), showing the thinning of tests in live compared to dead treated foraminifera (Figure 3C), while the non-elevated and moderately elevated $p\text{CO}_2$ treatments had little evidence of thinning when

TABLE 2 Comparison of test thicknesses between each treatment to the untreated control.

Treatments (pCO ₂)	Live treatment		Dead treatment	
	Treated vs. Untreated	Individual variance	Treated vs. Untreated	Individual variance
Non-Elevated	0.001	0.283	0.002	0.312
Moderately Elevated	0.005	0.300	0.000	0.313
Highly Elevated	0.023	0.365	0.014	0.293

Effect size (η^2) was calculated using two-way ANOVA models that account for both the treatment factor and the variations among individual foraminifera.



comparing the live and dead treatments (Figures 3D,E). Comparisons on each of the eight newest chambers also revealed thinner tests among the live compared to the dead foraminifera, particularly, under the high pCO₂ treatment. Statistical comparison of test thicknesses between live and dead treated specimens were visualized for each chamber by their η^2 values (Figure 4). Interestingly, higher effect sizes ($\eta^2 > 0.1$) were observed in the six newest chambers (from n to n–5), while a lower effect ($\eta^2 < 0.1$) was seen in chambers n–6 and n–7 among the high pCO₂ treatment of live versus dead specimens (Figure 4A).

Discussion

Calcareous foraminifera serve as carbon sinks across the global ocean by incorporating calcium carbonate to their tests, sequestering carbon from the surrounding seawater. Benthic foraminifera play important roles in the worldwide carbon budget with an estimated

production of 200 million tons of calcium carbonate per year (Langer, 2008). However, calcium carbonate production by foraminifera could be negatively impacted by the anthropogenic production of excess CO₂, which causes ocean and coastal acidification, subsequently decreasing the saturation of the carbonate system in the marine environment.

Ocean and coastal acidification could have mixed impacts on foraminifera, with studies noting that some foraminifera species can survive in moderate elevation of pCO₂ (790–1865 μ atm) without major growth defects (Schmidt et al., 2014; Haynert et al., 2014) or even showing increased growth rates (Fujita et al., 2011). However, the majority of studies indicate either decreased growth rate or defects in morphology at decreased pH (7.4–7.9) or at moderate to highly elevated pCO₂ (e.g., up to 3,247 μ atm) (Khanna et al., 2013; Prazeres et al., 2015; Schmidt et al., 2014; Keul et al., 2013; Haynert et al., 2014; Kuroyanagi et al., 2021; Manno et al., 2012; Kuroyanagi et al., 2009; Fujita et al., 2011; McIntyre-Wressnig et al., 2013; Raymond et al., 2013; Iwasaki et al., 2019;

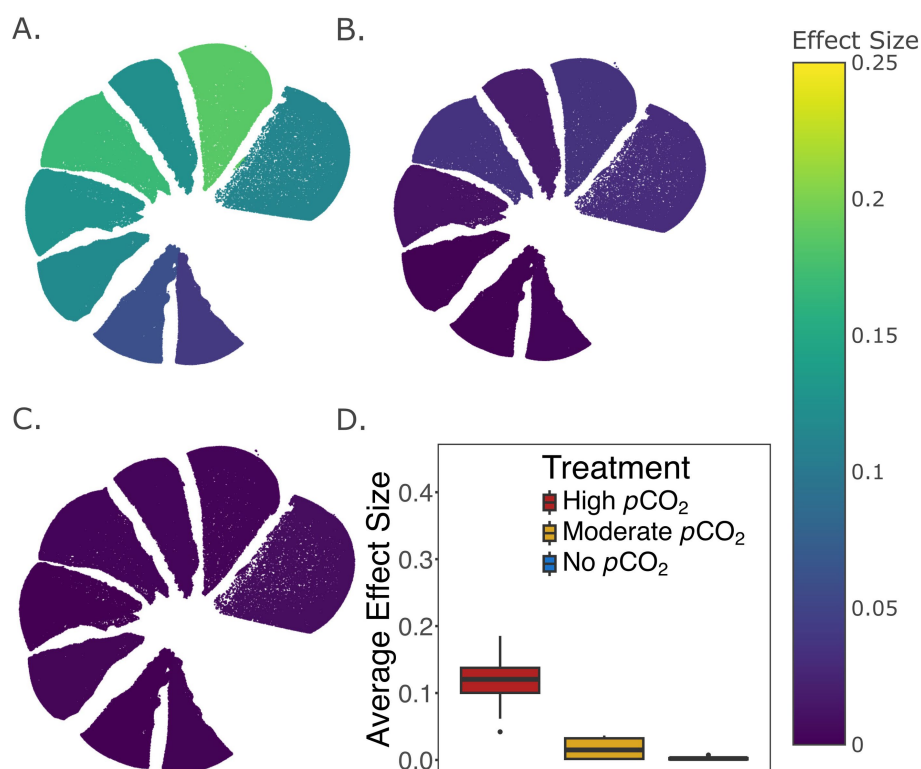


FIGURE 4

(A–C) Comparison of test thicknesses between live and dead treated specimens within each of the 8 newest chambers under the highly elevated $p\text{CO}_2$ (A), moderately elevated $p\text{CO}_2$ (B), and no elevated $p\text{CO}_2$ (C) treatment conditions. The color of each chamber represents the effect size (η^2) of the live vs. dead factor in a two-way ANOVA that accounts for variations among individual foraminifera. (D) Box and whisker plot showing the median, the first and third quartiles, and the minimum and maximum of effect sizes across all 8 chambers for each treatment. Only microspheric foraminifera were used in this analysis (Materials and Methods). The total number of specimens in each treatment group is documented in [Supplementary Table S3](#).

[Sinutok et al., 2011](#)). Study of *Haynesina germanica*, a temperate salt marsh foraminifer closely related to the *Haynesina* sp. examined in this study, suggests their feeding-related test ornamentation can be deformed during prolonged treatments (36 weeks) of moderately elevated $p\text{CO}_2$ (380–1,000 ppm) ([Khanna et al., 2013](#)). However, morphological alteration has not been systematically documented throughout the entire test. Further, with the projected increases of ocean $p\text{CO}_2$, more extreme acidification conditions, such as those observed in porewaters of estuarine mudflat sediments ([Fouet et al., 2024](#)), will become more impactful to coastal foraminifera.

To our knowledge, this is the first study that differentiates the two alternative generations of the foraminifera lifecycle, microsphere and megalosphere, in examining foraminifera responses to acidification. This distinction could be crucial as varied test structures have been observed between the two life stages of foraminifera, such as those documented in some Elphidiids ([Gudina and Levitchuk, 1989](#)). This variability is shown in our experimental data, where microspheric and megalospheric foraminifera had varied test thickness distributions and different levels of sensitivity to laboratory treatments ([Supplementary Figure S2](#)). Our current technology, however, supports the identification of life stages only after experimental treatments because of the destructive nature of extended exposure to high X-rays during MicroCT scanning (Materials and Methods). As a result, an insufficient number ($n < 3$) of megalospheres was included

in some treatment conditions ([Supplementary Table S3](#)), and the test-thickness analyses were performed only on microspheres. Therefore, the response of megalospheres to coastal acidification remains unknown, which could be a topic for future investigations, for example, by increasing the sample size of each treatment condition to increase the chance of capturing a sufficient number of both micro- and megalospheric specimens.

Compared to the untreated group, the experimental treatment of both live and dead microspheric foraminifera had a larger effect size ($\eta^2 > 0.01$) in highly elevated $p\text{CO}_2$ relative to the little to no effect ($\eta^2 \leq 0.005$) in non- or moderately elevated $p\text{CO}_2$ ([Table 2](#)). Most calcareous foraminifera form tests that are mainly composed of calcite, which is structurally more stable ([Erez, 2003](#)) and less prone to dissolution ([Sulpis et al., 2022](#)) than the calcium carbonate polymorph aragonite. Given that calcite oversaturation was measured in the moderate treatment ($\Omega_{\text{calcite}} = 1.498 \pm 0.057$), it is unsurprising that *Haynesina* test thickness exhibited little to no change in the moderately elevated $p\text{CO}_2$. In contrast, the highly elevated $p\text{CO}_2$ treatment exhibited calcite undersaturation ($\Omega_{\text{calcite}} = 0.843 \pm 0.041$), consistent with the observation of test thinning in both live and dead specimens ([Tables 1, 2](#)). It is worth noting that the treatment period of our study was 4 weeks, significantly shorter than the long-term treatment (36 weeks) performed on *Haynesina germanica* ([Khanna et al., 2013](#)). This shorter time frame was chosen because it better represents the environmental condition of the *Haynesina* foraminifera

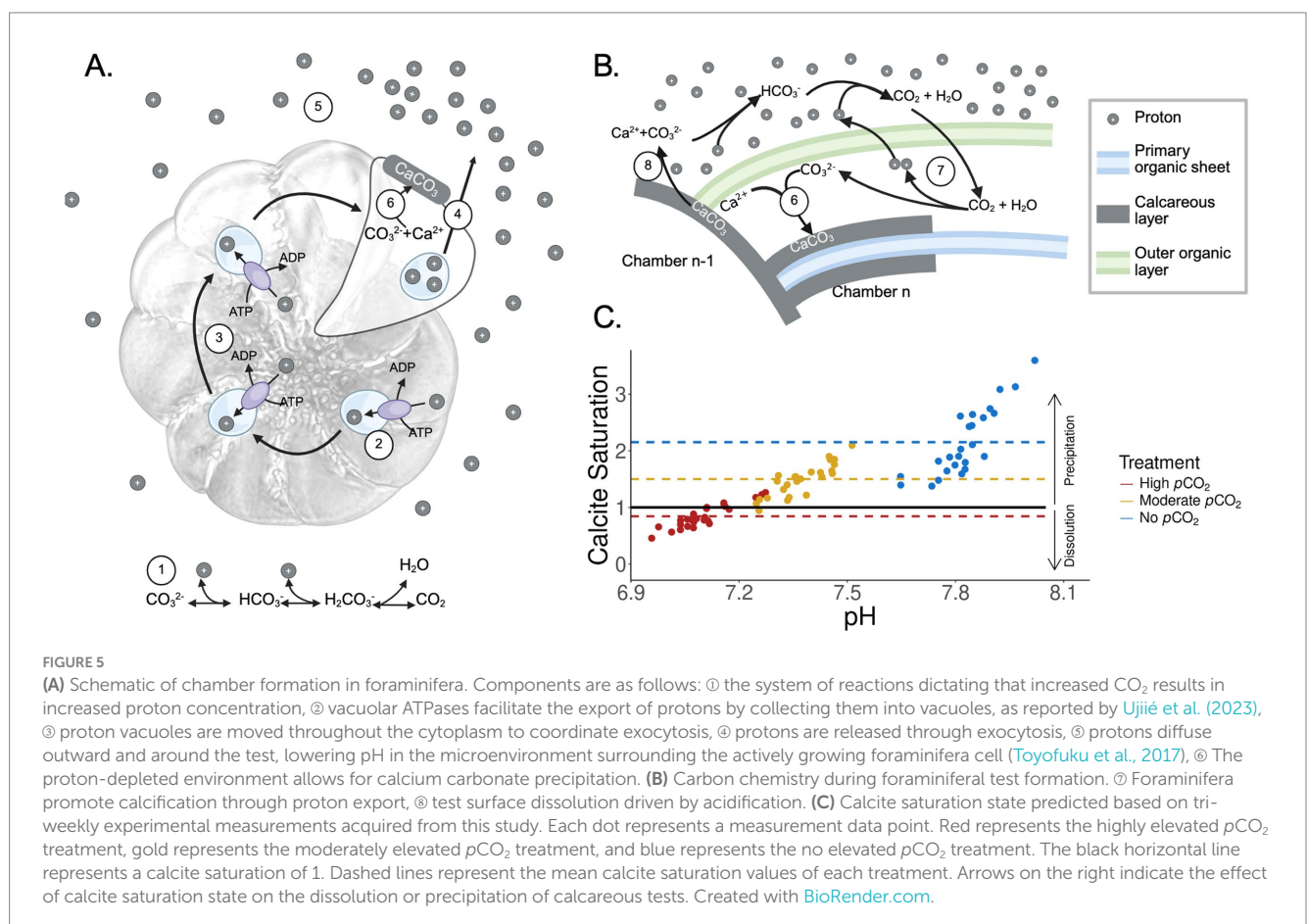
used in our study, where only short episodes of extreme low pH occur during summer months (Wallace et al., 2014). Future studies are required to examine acidification responses through extended periods under both moderately and highly elevated $p\text{CO}_2$, especially as such prolonged exposure becomes relevant to the coastal benthic environment.

Typically, new chambers in foraminifera precipitate via multiple steps: (1) formation of an outer organic layer, which is a protective cytoplasmic envelope that defines the bound of the new chamber; (2) construction of the primary organic sheet, which forms under the protective envelope; and (3) calcification around the organic sheet (Erez, 2003; Sliter, 1970; Toyofuku et al., 2017; De Nooijer et al., 2009; Nagai et al., 2018). The calcification relies on the maintenance of a local environment within the protective envelope with conditions favorable for calcium carbonate precipitation (Toyofuku et al., 2017; De Nooijer et al., 2009; Bentov and Erez, 2006; Bentov et al., 2009). This process could be facilitated by vacuolar ATPases, which transport protons from the calcification site to vesicles that are then exported to the extracellular space (Ujiié et al., 2023). Therefore, maintenance of calcification-promoting conditions in foraminifera could involve potential energetic expenses due to consumption of ATP for proton export.

Comparing the acidification treatment of live or dead specimens demonstrated differences in their responses to acidification, with the highest effect observed under the high $p\text{CO}_2$ condition (Figure 3). This indicates that thinning of foraminifera tests could be driven not only

by calcite undersaturation, but also by the physiological activity of live foraminifera, likely related to the formation of new chambers (Figure 2C). The chamber-specific comparison of live and dead specimens has further emphasized the significant effect of foraminifera physiology on test chamber thickness under highly elevated $p\text{CO}_2$. In particular, a more substantial effect size (η^2 from 0.11 to 0.19) was observed in each of the six newest chambers (n to n-5) compared to chambers n-6 ($\eta^2 = 0.06$) or n-7 ($\eta^2 = 0.04$) (Figure 4), suggesting potential effects of new chamber formation in exacerbating test thinning in high $p\text{CO}_2$ systems, likely due to the export of protons mediated by vacuolar ATPases.

Proton release during the formation of new test chamber can lead to increased proton concentration (Figure 5A), subsequently lowering the pH in the microenvironment that surrounds the foraminifera test (Toyofuku et al., 2017). The decreasing pH alters calcite saturation (Ω_{calcite}), which in turn can lead to potential dissolution of the test surface (Figure 5B). Under the no-elevated and moderately elevated $p\text{CO}_2$ treatments performed in this study, Ω_{calcite} is relatively high, and hence the decrease of pH caused by calcification could have less effect. However, Ω_{calcite} in the highly elevated $p\text{CO}_2$ was close to the value of 1 (Figure 5C), below which dissolution is expected due to calcite undersaturation. Therefore, even a slight decrease of pH could have significant effects on the foraminiferal test, not only increasing the energy demands in promoting calcification and new chamber formation, but also resulting in the dissolution of existing test surfaces.



We suggest that newer chambers could be more sensitive to acidification than the older chambers, as the physiologically driven pH reduction is likely initiated in the extracellular space near the site of calcium carbonate precipitation of the new chamber (Figure 5). Our experimental observations of the *Haynesina* sp. (Figure 4A) support models of foraminifera calcification previously described in other studies (Toyofuku et al., 2017; Ujiie et al., 2023) and are consistent with observations from another foraminifera, *Ammonia* sp., where the lowest extracellular pH in its surrounding microenvironment was measured near the newest chamber (Toyofuku et al., 2017; Glas et al., 2012).

The ability of foraminifera to use proton pumping to manipulate carbonate chemistry is a competitive advantage against ocean and coastal acidification, as it enables the organism to decouple calcium carbonate precipitation from the chemistry of the surrounding seawater (Toyofuku et al., 2017; De Nooijer et al., 2009; Bentov et al., 2009; Ujiie et al., 2023; Glas et al., 2012). However, our results suggest that in conditions near the borderline of calcite undersaturation, foraminifera could reach a tipping point that exacerbates the risk of test dissolution. Further, the energetic cost of proton pumping could increase with any continued rise of $p\text{CO}_2$ (De Nooijer et al., 2009), as foraminifera must overcome stronger concentration gradients to achieve an optimal calcification rate (Riebesell and Tortell, 2011). This is notable, as the $p\text{CO}_2$ conditions tested in this study have already been observed in coastal systems (Wallace et al., 2014). Therefore, coastal benthic foraminifera are likely experiencing acidification stress that impairs new chamber formation and dissolves already formed test surfaces. With continued anthropogenic production of CO_2 , coastal acidification will accelerate in intensity and duration (Findlay and Turley, 2021), leaving the ecological function of foraminifera as a carbon sink at greater risk.

Conclusion

This study used laboratory experiments to examine the morphological response of specimens belonging to the foraminiferal genus *Haynesina* to increasing acidification. The impact of moderate and high acidification conditions on the thickness of foraminifera tests were quantified, demonstrating potential resilience to moderate acidification but deficiency when experiencing high acidification. Contrasting responses of live versus dead foraminifera specimens further documented the combined effects of acidification and physiological processes in the thinning of test surfaces. These observations suggest that increasing ocean and coastal acidification will likely aggravate the precipitation deficiency of foraminifera and presumably other calcifying organisms.

Materials and methods

Field sampling and sample preparation

Surface sediments were collected into 125 mL high density polyethylene Nalgene containers using a plastic scoop. Collected samples were sieved with USA standard sieves 120 (Thermo Fisher Scientific 039988.ON) and 40 (Thermo Fisher Scientific 039984.ON) to select for the size fraction between 125 μm and 425 μm . Isolated sediments were subsequently picked for approximately 600

specimens of *Haynesina* sp. using 50 μL calibrated pipettes (Drummond Scientific Company 2-000-050), which were pulled to a thin point over a bunsen burner, to isolate individual foraminifera while visualizing with a trinocular stereo microscope under 10–25 $\times$ with maximum brightness (VanGuard 1372ZL). A subsample of 60 individuals were placed in 2 mL 6% sodium hypochlorite solution (Fisher Scientific NC1796686) for 12 h to remove organic material from the test through bleaching. After bleaching, specimens were rinsed twice for five minutes with Milli-Q H_2O (Type I H_2O purified with EMD Millipore MilliQ EQ-7008). Eight of the bleached specimens were collected as a no-treatment control (i.e., untreated) and were retained in 100% ethanol at 4°C until microscopic imaging. The rest of the bleached specimens were used in the dead treatment and were stored in Milli-Q H_2O at 4°C until experimental manipulation. The rest of the picked foraminifera were kept alive under room temperature in artificial seawater composed of Milli-Q water and Reef Pro Mix (Fritz Aquatics 80,243) made at a salinity of 35 ppt until being used as live treatment in experimental manipulation.

Experimental $p\text{CO}_2$ manipulation

Experimental $p\text{CO}_2$ manipulation was performed in three 75-liter glass treatment tanks with target pH maintained at 7.2 (Tank 1), 7.6 (Tanks 2), and 8.1 (Tank 3), respectively. All treatment aquaria were maintained with artificial seawater. Replicate foraminifera samples were introduced to treatment tanks in six-well plates sealed with 60- μm nylon mesh (Amazon ASIN#B092D8TJDQ). Each tank had 4 replicate six-well plates, with each plate contained 35 live foraminifera in one well and 3 bleached (dead) foraminifera in a separate well. The acidification treatments were designed following prior examples (Putnam et al., 2016), with $p\text{CO}_2$ levels controlled using an A3 Apex Aquarium Controller System (Bulk Reef Supply, SKU 251246). The Apex system measures pH and temperature (°C) every 10 s and adjusts the pH to a target value by injecting CO_2 gas using controls of solenoid valves (Figure 1A). Three times per week (Monday, Wednesday, and Friday), 200 mL of tank water from each glass tank were filtered through 0.2 μm surfactant-free cellulose acetate (SFCA) syringe filters (Thermo Scientific 723–2,520). This filtered seawater was stored at –20°C for stability before being used for carbonate system analysis (Mos et al., 2021). At each time point, pH was measured using a calibrated pH meter (OHAUS Aquasearcher 30,589,830), salinity was measured using a refractometer (Amazon ASIN#B018LRO1SU), and temperature was measured using the Apex controller temperature probe (Bulk Reef Supply, SKU 207517). Each Friday, the OHAUS pH meter was calibrated through examination of temperature and voltage correlation, and replicate wells of live foraminifera specimens were fed with *Skeletonema dohrnii* clone PA 250716_D1 isolated from Narragansett Bay and obtained from Dr. Tatiana Rynearson's lab at the University of Rhode Island Graduate School of Oceanography. The *S. dohrnii* was cultured in F/2 medium (Guillard and Ryther, 1962) under 12 h light and 12 h dark cycles. At the time of feeding, concentration of live *S. dohrnii* culture was quantified with a hemocytometer (Fisher Scientific 02-671-6) to determine the volume used for feeding live foraminifera. An average of 124 μL *S. dohrnii* culture was used in each feeding to add approximately 25,000 cells to each treatment.

At the end of the experimental period (28 days), a subset of the specimens from both treatments ($n_{\text{live}} = 6-8$, $n_{\text{dead}} = 10-12$) were randomly collected and prepared for MicroCT scanning. Samples of experimentally treated live specimens were bleached in 6% sodium hypochlorite solution (Fisher Scientific NC1796686) for 12 h to remove organic material from the test. The bleached tests from both live and dead cell treatments were washed twice with Milli-Q water, followed by subsequent washing with 50, 80, and 100% ethanol to rinse any remaining debris and dehydrate the tests in preparation for microscopic imaging ([Supplementary Figure S3](#)). All the live and dead treatment specimens were stored in 100% ethanol at 4°C until microCT scanning, which facilitated air drying the tests for mounting.

Seawater carbonate chemistry

Filtered tank water stored at -20°C was used for carbonate-system analysis. Quality control for pH data was assessed three times per week with Tris standard (Dickson Lab Tris Standard Batch 205) and handheld conductivity probes used for discrete measurements were calibrated once per week. Total alkalinity (TA) was measured using an open-cell titration ([Dickson et al., 2007](#)) with certified HCl titrant ($\sim 0.1 \text{ mol kg}^{-1}$, $\sim 0.6 \text{ mol kg}^{-1}$ NaCl; Dickson Lab) and TA measurements identified < 1% error when compared against certified reference materials (Dickson Lab CO_2 CRM Batch 196). Seawater chemistry was completed following Guide to Best Practices ([Dickson et al., 2007](#)). Tri-weekly measurements were used to calculate carbonate system parameters ([Table 1](#)), using the SEACARB package ([Gattuso et al., 2015](#)) in R v3.5.1.

Imaging of foraminifera tests with microCT scanning

Foraminifera tests (untreated, dead treated, and live treated) preserved in 100% ethanol were allowed to air dry completely before mounted with Bondic resin (Bondic, CECOMINOD032561) on a flat surface and cured under UV light. Coordinates of mounted tests were identified through a prescan with a Zeiss Xradia Versa 610 X-Ray microscope under the 0.4x objective using the following parameters: 50 kV voltage, 4.5 W power, 401 projections. Identified tests were individually imaged with the following imaging parameters under the 4x objective: 80 kV voltage, 10 W power, 2,401 projections. Stacked TIFF images were produced based on automatic reconstruction settings during the imaging. The resulting image stacks were imported into the Dragonfly image analysis software (ORS systems Core dll version 2022.2.0.1399, Montreal, CA), which creates a 3D-reconstruction for each foraminifera test. The 8 newest chambers in the 3D-reconstruction of each test were manually isolated through the graphical interface of the Dragonfly software by extracting a region of interest (ROI) containing test areas that are visible from the outside and deleting any undesired regions (e.g., the sutures or air bubbles introduced by the mounting process) ([Figures 1B,C](#)). Voxels with an intensity lower than 32,000 were filtered out from each chamber, preserving regions that contained the calcium carbonate test, but excluding voxels that imaged the resin or most air bubbles. The extracted ROIs were then used to calculate a thickness mesh using the “generate thickness mesh” function in Dragonfly, where thicknesses

throughout the test were calculated by fitting spheres between the outer and inner test surfaces. The thickness mesh of each test chamber was individually exported to a csv file and used for statistical analysis. The number of thickness measurement data points exported ranged between 49,721 and 700,539 per chamber, covering the entire ROI of each chamber.

Classification of microspheric and megalospheric foraminifera

Diameter of the proloculus and the overall test were determined by fitting a smallest possible sphere over their corresponding outer surfaces using the Dragonfly image analysis software, where the radius of the fitted sphere was reported and used for calculating the diameter of its corresponding proloculus or test. The number of chambers present in each foraminifera was manually counted based on an internal slice projection that included all chambers. During analysis of the 3D-reconstruction of foraminifera tests, a bimodal distribution of proloculus diameters were observed, resulting in two populations: (1) megalospheric, tests with proloculus diameter greater than or equal to $55 \mu\text{m}$, (2) microspheric, tests with proloculus diameter less than $55 \mu\text{m}$ ([Supplementary Figure S1](#)). Correspondingly, these two populations had distinct distributions of the number of chambers ([Figure 2B](#)).

Data analysis

All statistical analysis was performed in R v4.2.3 using the sjstats package version 0.19.0 ([Lüdtke, 2024](#)) and the stats package version 4.2.3. Results were visualized in R v4.2.3 using ggplot2 version 3.4.1 and plotly version 4.10.4 ([Ginestet, 2011](#); [Plotly Technologies Inc, 2015](#)). The number of chambers per test and the test diameters were compared between microspheric and megalospheric foraminifera using one-way analysis of variance (ANOVA) ([Figure 2B](#)). Growth of live foraminifera throughout the treatment period was approximated by comparing their number of chambers to the pool of dead treated and untreated specimens (referred to as “not-live”) using two-way ANOVA that accounted for differences in microspheric and megalospheric samples, followed by the Tukey’s honestly significant difference test (TukeyHSD) ([Figure 2C](#)). Data normality was confirmed through inspection of quantile-quantile plots before the application of ANOVA models.

Due to the low abundance of megalospheric specimens in several treatments ([Supplementary Table S3](#)), all the statistical analyses related to test thicknesses were performed with only the microspheric foraminifera. To normalize the thickness measurements from microCT scanning, test thicknesses were divided by the diameter of each corresponding test. The normalized thickness values were compared using two-way ANOVA that accounted for a treatment factor (e.g., treated versus untreated, different $p\text{CO}_2$ conditions, or live versus dead treatment) and a second factor that accounted for variations of individual foraminifera. To account for the large number of thickness measurement data points from each specimen, all ANOVA analyses that showed statistical significance ($p < 0.05$) were followed by the calculation of effect size (η^2) measures ([Table 2](#); [Figures 3, 4](#)). The effect size ranges from 0 to 1 and is representative of the proportion of variance in the model explained by a given factor.

Specifically, test thickness differences between experimentally treated and untreated foraminifera were examined separately with live or dead specimens and across the three $p\text{CO}_2$ treatments (Table 2). Variation of test thicknesses across different $p\text{CO}_2$ conditions were compared separately for the live or the dead treatments (Figures 3A,B), and the variation between live and dead foraminifera were compared separately for the different $p\text{CO}_2$ conditions (Figures 3C–E). Finally, test-thickness variations between live and dead specimens were examined within each of the eight newest chambers (from n to n–7) to assess their differential responses to the different $p\text{CO}_2$ conditions (Figure 4).

Data availability statement

Data files including water chemistry data and test thickness measurements are available on figshare at <https://figshare.com/s/4464cc33548faf92e211>. All scripts used for analysis are available at <https://doi.org/10.5281/zenodo.15653269>.

Author contributions

CP: Conceptualization, Formal analysis, Methodology, Validation, Writing – original draft, Writing – review & editing, Data curation, Investigation, Software, Visualization. AP: Data curation, Investigation, Methodology, Writing – review & editing. AZ: Data curation, Investigation, Methodology, Writing – review & editing. KH: Data curation, Investigation, Methodology, Writing – review & editing. MG: Data curation, Investigation, Methodology, Writing – review & editing. JB: Investigation, Methodology, Writing – review & editing, Formal analysis, Validation. YZ: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Formal analysis, Methodology, Validation, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research and/or publication of this article. This project was supported by the National Science Foundation Office of Integrative Activities, award #1929078 and an Undergraduate Research Award from the University of Rhode Island (Fall 2022). The MicroCT data was acquired at the RI Consortium for Nanoscience and Nanotechnology, a URI College of Engineering core facility partially funded by the National Science Foundation EPSCoR, Cooperative Agreement #OIA-1655221.

Acknowledgments

We thank Dr. Tatiana Rynearson's laboratory for providing culture of *S. dohrnii* PA 250716_D1 for the feeding of live foraminifera during acidification treatments.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2025.1570629/full#supplementary-material>

SUPPLEMENTARY FIGURE S1

(A) Cross section of a microspheric test. (B) Cross section of a megalospheric test. Each scale bar represents 100 μm . (C) Histogram of proloculus diameters showing a bimodal distribution.

SUPPLEMENTARY FIGURE S2

(A–E) Distribution of normalized test thickness between microspheric and megalospheric specimens within each experimental treatment.

SUPPLEMENTARY FIGURE S3

Schematic of experimental workflow as detailed in materials and methods. Created with BioRender.com.

SUPPLEMENTARY TABLE S1

Collection of previous ocean and coastal acidification studies of foraminifera, including reference information for the paper, condition of acidification treatments (i.e., pH, $p\text{CO}_2$), and a summary of key findings.

SUPPLEMENTARY TABLE S2

Tri-weekly water chemistry data from each treatment tank of both the Summer 2023 and Fall 2023 experiments. Parameters shown in the table were calculated using the SEACARB R package V3.5.1 with the exception of pH, temperature, salinity, and total alkalinity (TA), which were experimentally measured.

SUPPLEMENTARY TABLE S3

Number of megalospheric and microspheric specimens collected from the Summer 2023 and Fall 2023 acidification experiments across each treatment. Total indicates the sum of numbers from both replicate experiments.

SUPPLEMENTARY TABLE S4

Measurements of foraminifera test morphology for all specimens analyzed in this study. Data includes the trial number (OA2 or OA3), treatment tank, specimen ID, measurements of test radius/diameter, proloculus radius/diameter, number of chambers, and assignment of foraminifera life stage.

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