ECOLOGY

Specific host-algae relationship, yet flexible bacterial microbiome, in diatom-bearing foraminifera

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Whether the adaptive strategies of marine mixotrophs, organisms that combine heterotrophic and autotrophic nutrition, in response to global change are rooted in their symbiotic relationships is debated, especially for larger benthic foraminifera. Despite their importance in the ecosystem, there are controversial findings regarding the specificity of their algal endobionts, preventing a deeper understanding of their adaptive strategies. Using single-cell metabarcoding on 243 diatom-bearing foraminifera specimens from Indonesia, we found one highly dominant diatom strain in each foraminiferal host species bearing at least 90% of the reads in a majority of host species, whereas the bacterial community was very flexible, with only 25% of the variation explained by water depth, substrate type, location, and host species. Our results suggest that the adaptive strategy of the foraminiferal holobiont rather lies within its bacterial endobiome. Its dynamism likely facilitates the adaptive potential of foraminifera, supporting their proliferation across different environmental settings.

INTRODUCTION

Marine organisms have evolved multiple strategies to survive and thrive in nutrient-scarce ocean regions (*1*–*3*). One particularly well-adapted group of organisms to such oligotrophic environments are mixotrophs, organisms that combine photosynthesis and phagotrophic nutrition, the engulfment of nutritive particles, to obtain resources (especially carbon, nitrogen, phosphorus, and iron) necessary to sustain themselves (*4*, *5*). Several ancestrally heterotrophic organisms acquired the potential for mixotrophic nutrition by hosting photosynthetic endosymbionts (*4*, *6*), a strategy to integrate new metabolic functions (*7*). These symbioses, often obligate, enable efficient resource use and internal nutrient recycling (*6*, *8*), ideal in oligotrophic environments such as coral reefs. However, changing environmental conditions can trigger a shift in the relative importance of feeding versus photosynthesis of mixotrophic organisms in the ecosystem (*9*) and be detrimental to their survival by disrupting symbioses from mutualistic to neutral or parasitic ones (*10*, *11*). One well-known example of such a harmful phenomenon to the host is the disruption of the symbiosis leading to bleaching (*12*), reported worldwide in corals (*13*) and large benthic foraminifera (LBF) (*14*). It is, therefore, crucial to better understand the intricacy of these mixotrophic symbioses to foreshadow their sensitivity to future climatic conditions (*15*). This knowledge is especially relevant considering that most of marine mixotrophs are key organisms to ecosystem functioning by contributing largely to the ocean's carbon fixation and sequestration (*16*, *17*).

Many mixotrophs are found among protists, eukaryotic unicellular organisms, and those that host endosymbionts are very abundant and globally distributed across oceans (*18*). Mixotrophic marine protists are very successful due to their generalistic nature, likely giving them an advantage to adapt on the doorstep of changing environmental conditions (*5*). Despite their importance in the ecosystem, there is a marked lack of available datasets and knowledge of the holobiont composition as well as the understanding of adaptive strategies of marine mixotrophic protists. These important gaps are mainly due to the fact that protists are very small and overlooked in most ecosystems, additionally to having extraordinary and complex genetics (*19*, *20*).

Symbiont-bearing LBF are unicellular, mixotrophic, marine protists that excel as carbonate producers (*21*, *22*). Those LBF form a symbiosis with microalgae next to hosting a diverse prokaryotic community (*23*–*26*). Compared to the well-studied coral and plankton holobionts (*27*–*30*), relatively little research has focused on understanding these benthic mixotroph holobionts (*7*, *23*, *31*, *32*). Within the available literature, the specificity and selectivity of algal symbionts by LBF are subject to controversy. LBF have mostly been seen as "flexible" hosts simply because different foram taxa house different microalgal groups (i.e., rhodophyte, chlorophyte, diatom, and dinoflagellate) (*26*). Beyond that, histological, morphological, and molecular analyses revealed that single individuals can house a variety of symbiotic algal taxa, hinting toward a flexible foram-algae symbiotic relationship (*33*–*37*). Conflicting molecular results rather showed the dominance of one photosymbiont in single individuals, suggesting that LBF might be rather specific in their symbiotic algae relationship (*38*, *39*). Additionally, variations in the composition of the bacterial assemblages in LBF was shown to be triggered by water depth and surrounding microhabitat, underlining their flexibility within host species (*39*–*42*), while a number of bacterial taxa were found to be specific to the host species (*24*, *43*). Among those studies, only two studies compared the foraminifera microbiome to the environmental microbiome from the sediment (*24*) or seawater (*41*).

We aim to resolve the debates by bringing a complete dataset of the algal and bacterial microbiomes for six species of diatom-bearing foraminifera and shed light into the understanding of adaptive strategies of LBF to support their proliferation across a range of different environmental settings. We hypothesize that symbiotic diatoms are host specific, with one species dominating the endobiont community while being scarce in the environment, whereas the bacterial community within the host is more flexible and reflects the

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composition of the environmental microbiome. To test the hypothesis, we used single-cell metabarcoding from the foraminiferal holobiont to study the microalgal and bacterial communities present within the foram host, which we define as endobiomes with no assumption of a positive, neutral, or negative relationship. We collected specimens from three LBF families across coral reefs along a gradient of turbidity in the Spermonde Archipelago, Indonesia, in the middle of the Coral Triangle, a marine biodiversity hotspot (Fig. 1). We integrated the algal dataset from Barnes (*44*) to our data, the only published study providing data comparable to ours. Our results confirm the species specificity of the algal endobiont communities in diatom-bearing foraminifera and suggest the bacterial endobiome to drive the host adaptation to environmental changes.

RESULTS

Foram host species as the greatest predictor for endobiome compositions

Diatom-bearing foraminifera (forams) endobiomes sampled across six host species, three sampling depths, and six islands (Fig. 1) along

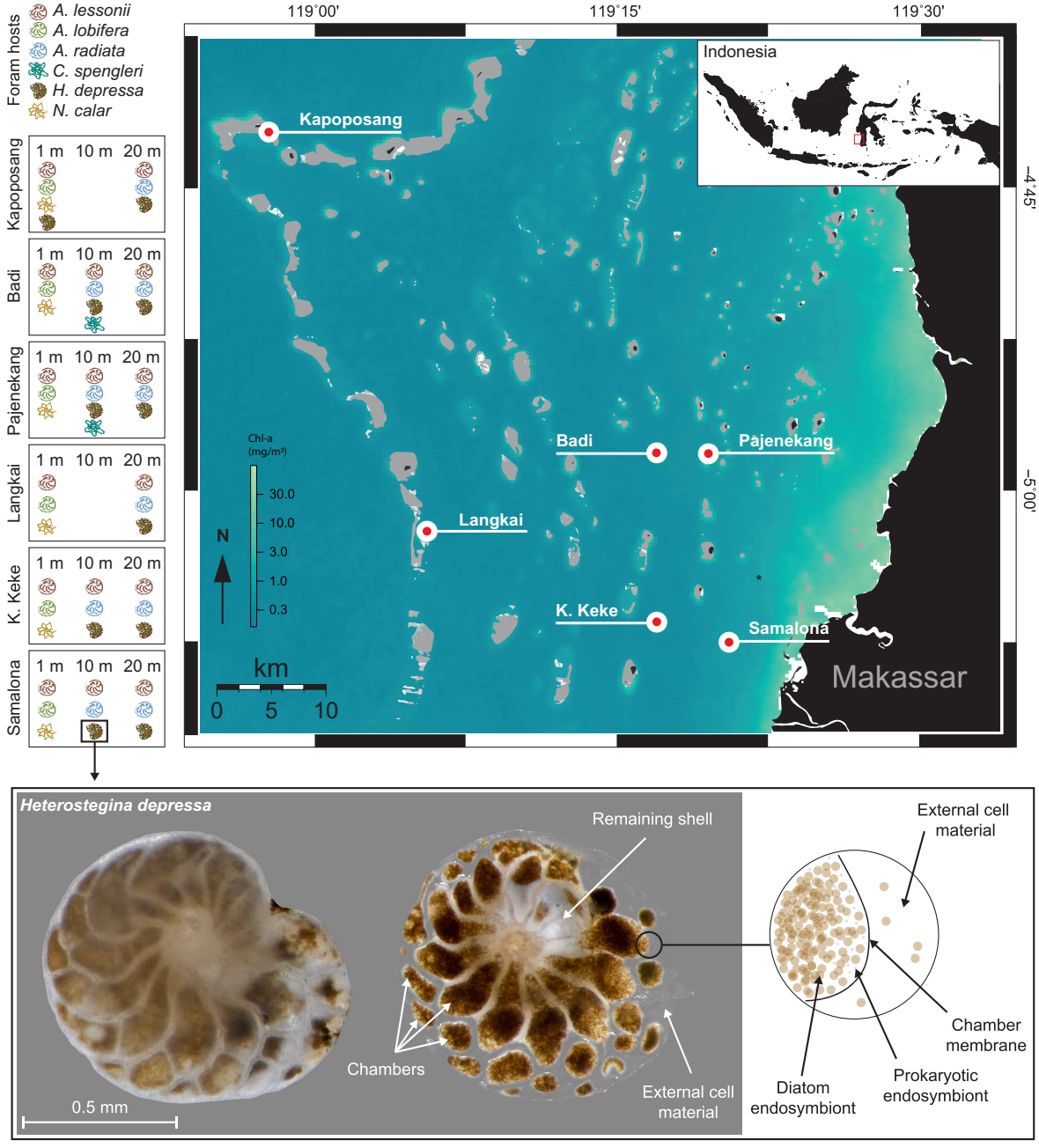

Fig. 1. Map of the Spermonde Archipelago and schematic representation of the endobionts. Sampling sites in the Spermonde Archipelago, South Sulawesi, Indonesia, with Sentinel 3 satellite image of the chlorophyll a (Chl-a; mean July 2022) following a near to offshore gradient. Sixteen sampling sites at six islands are displayed, indicating which host foraminifera species were analyzed. At the bottom: A specimen of *H. depressa*, diatom-bearing foraminifera, as a whole and partially decalcified, described with a schematic representation of its endobionts (from left to right).

a gradient of turbidity from the Spermonde Archipelago (South Sulawesi, Indonesia) resulted in a total of 1615 diatom and 2437 bacterial exact sequence variants (ESVs). We performed a redundancy analysis (RDA) to assess to what degree our variables (host species, depth, substrate type, and island location) explain the patterns in the diatom and bacterial endobiomes, both between host species (Fig. 2) and within host species (Table 1). The island location is used as a proxy for the change in the water quality along the gradient of turbidity in the Spermonde Archipelago. The foram host species was the greatest predictor of the diatom and the bacterial endobiome compositions (Fig. 2). Half of the variation in the diatom endobiomes was solely explained by the host species [RDA, 47.68%; analysis of variance (ANOVA), $P < 0.001$], while this was true for only a tenth of the variation in the bacterial species (RDA, 10.07%; ANOVA, $P < 0.001$). When performing the analysis only with the variables linked to the microhabitat, i.e., the local environment around each sampling site (depth, substrate type, and island location), the amount of variation in the bacterial endobiome explained by the model almost double (RDA, 17.60%; ANOVA, $P < 0.001$). Depth, substrate type, and island location contributed only a tenth to explain the variation in the diatom endobiomes (RDA, 10.63%; ANOVA, $P < 0.001$). The diatom endobiomes within *Amphistegina lobifera*, *Amphistegina radiata*, and *Neorotalia calcar* and the bacterial endobiomes within every host species were also significantly shaped by the island location (Table 1).

Weak core community across all foram host species

One way of testing for host specificity of endobiomes is by identifying a core community, which we define as an ESV present in >95% of the specimens considered across all host specimens, independently of the read abundance. There was no core diatom or bacterial endobiome identified across all 243 foram specimens. However, host-specific core diatom ESVs were detected in *Amphistegina lessonii*, *Heterostegina depressa*, *Calcarina spengleri* and *N. calcar* (fig. S1A). Host-specific core bacterial ESVs were also detected in *A. lessonii* and *C. spengleri* (fig. S1B). Overall, five diatom ESVs and five bacteria ESVs were present in more than 50% of all specimens (fig. S1, C and D). Most host species showed unique diatom and bacterial endobiomes, except for the endobiomes of *C. spengleri*, which were not significantly different from all other (in diatoms) and some other (in bacteria) host species endobiomes (Dunn's post hoc test, table S1).

Highly specific host-algae relationship

A simple overview of the 20 most abundant ESVs in the diatom and bacterial endobiomes revealed the community structure and the level of host specificity of those communities. Notably, the diatom endobiome of each host species was dominated by a single, highly abundant diatom ESV, except for *A. radiata*, which had two (Fig. 3A). Those ESVs accounted for more than 90% of the reads in all foram host species, except in *A. lobifera* (30 to 80%). Every host species had a strong relationship to one endobiont diatom species (>75% mean relative read abundance) in a majority of the specimens (Fig. 3A), referred to as the primary endobiont. *A. lessonii* and *A. lobifera* were populated by a diatom of the genus *Serratifera* (ESV2 and ESV5, respectively), *A. radiata* by *Talaroneis posidoniae* (<95% ID; ESV4 and ESV5), *C. spengleri* by *Nitzschia* cf. *microcephala* (ESV8), *H. depressa*

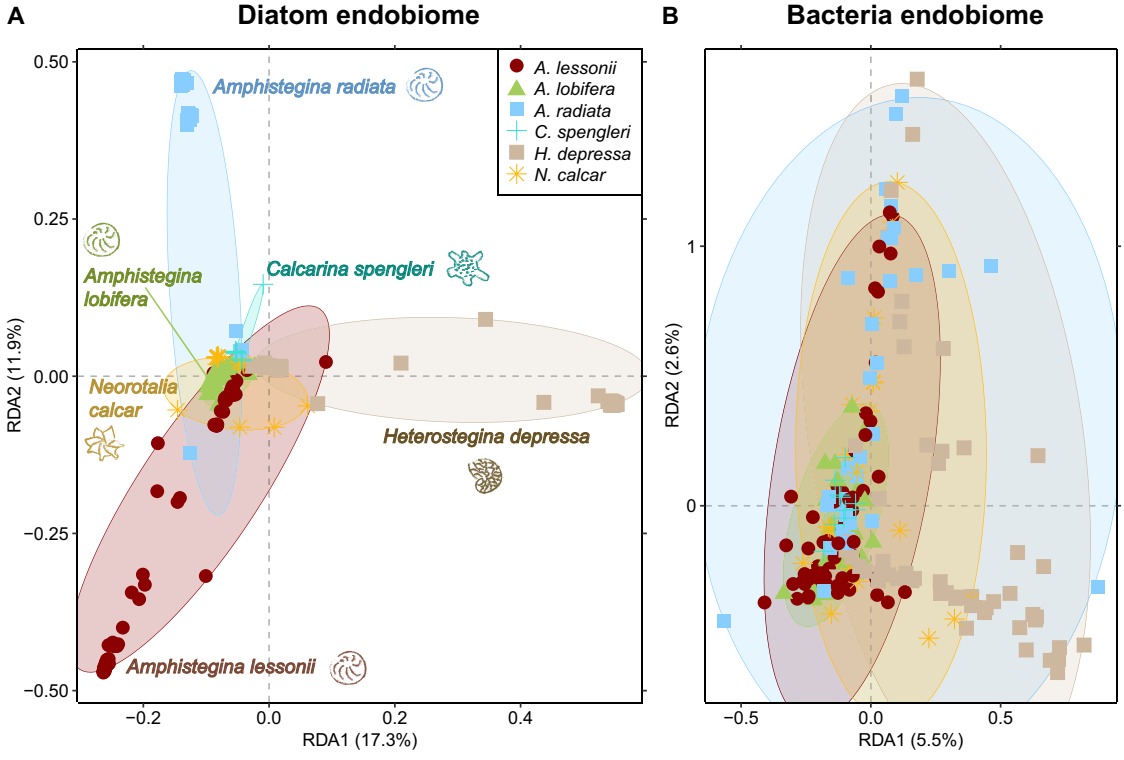

Fig. 2. Foram host species as predictor for endobiome compositions. Redundancy analysis (RDA), using Bray-Curtis dissimilarities, performed with foram host species as the sole predictor for the diatom (**A**) and the bacteria (**B**) endobiomes. Only the first two axes are displayed. Samples are coded by colors and shapes indicating the host species (dark red circles, *A. lessonii*; green triangles, *A. lobifera*; blue squares, *A. radiata*; turquoise crosses, *C. spengleri*; brown squares, *H. depressa*; orange stars, *N. calcar*). The ellipses show the 95% confidence interval for each host species.

Table 1. Redundancy analysis (RDA) performed on all host species and per host species. Analyzing all host species together and each host species individually, we assessed the impact of predictors linked to the microhabitat (water depth, substrate type, and island location) in shaping the diatom and bacterial endobiomes within foram host species. Island location is used as a proxy for the change in the water quality along the gradient of turbidity in the Spermonde Archipelago. Only the two first RDA axes are displayed here. ANOVA was used to assess the significance of the RDA model, the axes and the terms (predictors), marked with asterisks: *, 0.05; **, 0.01; ***, 0.001.

Endobiome	Host species	RDA	*P* value model	*P* value axes	*P* value terms
Diatoms	All together†	47.68%	0.001***	RDA1, 0.001*** RDA2, 0.001***	Host species, 0.001***
	All together‡	10.63%	0.001***	RDA1, 0.001*** RDA2, 0.033*	Substrate, 0.002** Depth, 0.001***
	A. lessonii	13.99%	0.3	All, >0.7	All, >0.083
	A. lobifera	23.76%	0.022*	All, >0.093	Island, 0.021*
	A. radiata	42.63%	0.002**	RDA1, 0.001***	Island, 0.001***
	C. spengleri	18.21%	0.139	All, >0.1	All, >0.1
	H. depressa	20.05%	0.207	All, >0.2	All, >0.069
	N. calcar	46.12%	0.008**	RDA1, 0.004**	Island, 0.002**
Bacteria	All together†	10.07%	0.001***	RDA1, 0.001*** RDA2, 0.001***	Host species, 0.001***
	All together‡	17.60%	0.001***	RDA1, 0.001*** RDA2, 0.003***	Island, 0.001*** Substrate, 0.001*** Depth, 0.001***
	A. lessonii	27.87%	0.001***	RDA1, 0.001***	Island, 0.001*** Substrate, 0.024* Depth, 0.002**
	A. lobifera	31.40%	0.001***	RDA1, 0.001*** RDA2, 0.002**	Island, 0.001***
	A. radiata	47.13%	0.001***	RDA1, 0.001*** RDA2, 0.006**	Island, 0.001*** Substrate, 0.001***
	C. spengleri	53.96%	0.017*	RDA1, 0.011*	Island, 0.007**
	H. depressa	33.24%	0.001***	RDA1, 0.001*** RDA2, 0.004**	Island, 0.001***
	N. calcar	49.86%	0.001***	RDA1, 0.001*** RDA2, 0.001***	Island, 0.001***

†RDA performed on all host species together using only host species as predictor (term). ‡RDA performed on all host species together using water depth, substrate type, and island location as predictors (term).

by *Thalassionema frauenfeldii* (ESV1), and *N. calcar* by *Nitzschia inconspicua* (ESV6). We performed a species occupancy modeling to test the observed host specificity from the community composition. Results from the model showed that the primary diatom endobiont was more likely to be found in that specific host than in the environment ($P < 0.01$) (fig. S3). The diversity indices of the host-associated diatom endobiomes supported this notable prevalence, with a low Pielou evenness value, a low Simpson's index (model 1-D) value, and a low diversity based on the Shannon-Wiener index (fig. S2). Despite the primary diatom species dominating the endobiome, its abundance still varied between specimens, especially in the host species *A. lobifera* (Fig. 3A and fig. S1A). Additionally, between 6 and 33% of the specimens had an abundant diatom endobiont different from the primary endobiont of the host species (fig. S4).

To grasp the foram-diatom relationship, we analyzed phylogenetic relationships between the primary endobiont diatom species and between their respective host species, which were congruent with high bootstrap values (>70%) (Fig. 4). The amphisteginids and the calcarinids form each their own clade, so did their primary endobionts from the *Serratifera* and *Nitzschia* genera, respectively. *H. depressa* is a sister group to the calcarinids, similarly to its primary endobiont closest to the diatom species *T. frauenfeldii*, which is sister to *Nitzschia* (Fig. 4). However, most of the shallow branches were not well supported (bootstrap values of <70%). The endobiont diatom sequences of *A. lobifera*, *A. lessonii*, and *A. radiata* published by Barnes (*44*), from Hawai'i and Papua New Guinea, grouped within short distances to our primary diatom sequences for the respective foram hosts. In addition, most of Barnes' *A. radiata* diatom clones (Papua New Guinea) are identical to the diatom ESVs we sequenced from our Indonesian *A. radiata* samples, which were most similar (<95% ID) to the diatom *T. posidoniae*.

Flexible host-bacteria relationship

Contrasting with the diatom endobiomes, the bacterial endobiomes did not have extremely abundant ESVs (fig. S1B), and its diversity was much higher than that of diatoms per specimen (fig. S2). The 20 most abundant bacteria ESVs had lower overall relative

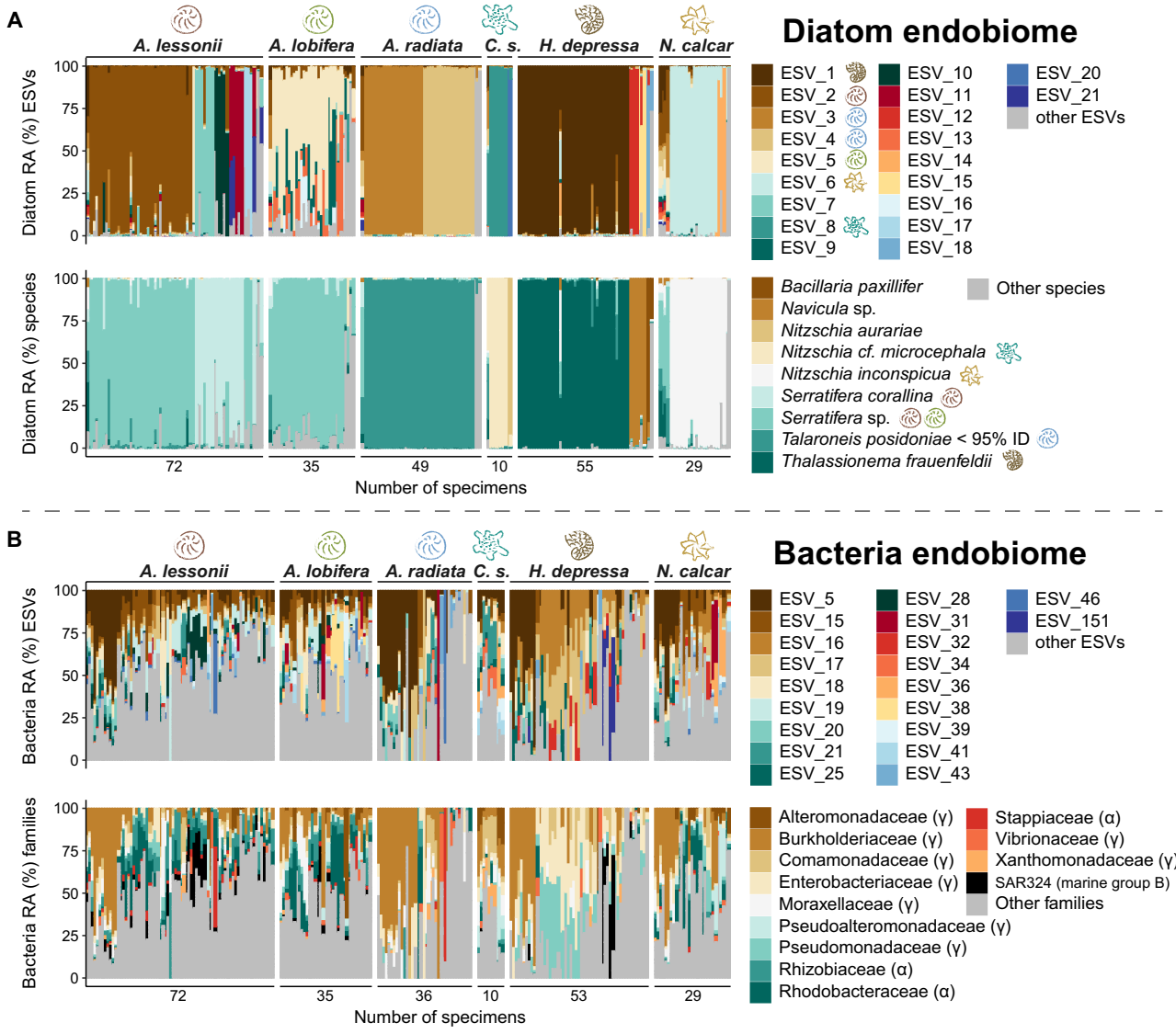

Fig. 3. Endobiome compositions across the six host species. Relative read abundance (RA) of the 20 most abundant diatom (**A**) and bacterial (**B**) ESVs. The bottom panel of each facet displays the taxonomic assignment of the ESVs, where the diatoms are color coded at the species level, and the bacteria at the family level (class of Proteobacteria in parentheses). The remaining composition of the endobiomes was grouped into "other," marked in gray in all panels. Note: The four panels have their own legends, and colors are not correlated between panels. Each host foraminifera species has its own logo, which is used to highlight the primary diatom endobiont in the legend (A).

abundances (5 to 35%), with a great proportion of the diversity contributed by low abundance ESVs (Fig. 3B and fig. S1B), and were more evenly distributed across foram host species compared to the diatom endobiomes (Figs. 2B and 3B). The majority was assigned to the class Gammaproteobacteria and, to a lesser extent, to Alphaproteobacteria (Fig. 3B). The flexible nature of the bacteria endobiome was supported by the diversity indices demonstrating the evenness of the endobiome and the absence of primary bacteria endobionts (fig. S2). The number of bacterial ESVs shared between specimens of the same host species from a single microhabitat was significantly higher than from different microhabitats (fig. S5A). Similarly, the number of ESVs shared between two specimens of the same host species was significantly higher than between two specimens of different host species from the same microhabitat, in 56% of the studied sites (fig. S5B).

To better understand the foram-bacteria relationship, we performed a taxonomy-based analysis to predict functional roles of the bacterial community within host species and the environment. The bacterial endobiome had the same basic functional roles across all foram host species at all depths and islands. Four putative metabolic functions were highly represented: ammonia oxidation, nitrite reduction, sulfate reduction, and dehalogenation (fig. S6). A group of bacteria predicted to perform chitin and xylan degradation was less abundant in the endobiomes but was slightly more represented in *H. depressa* compared to that in other host species (fig. S6).

Limited presence of endobionts in the environmental microbiomes

We compared the host endobiomes with the environmental microbiome (water and sediment) to confirm the level of specificity of the

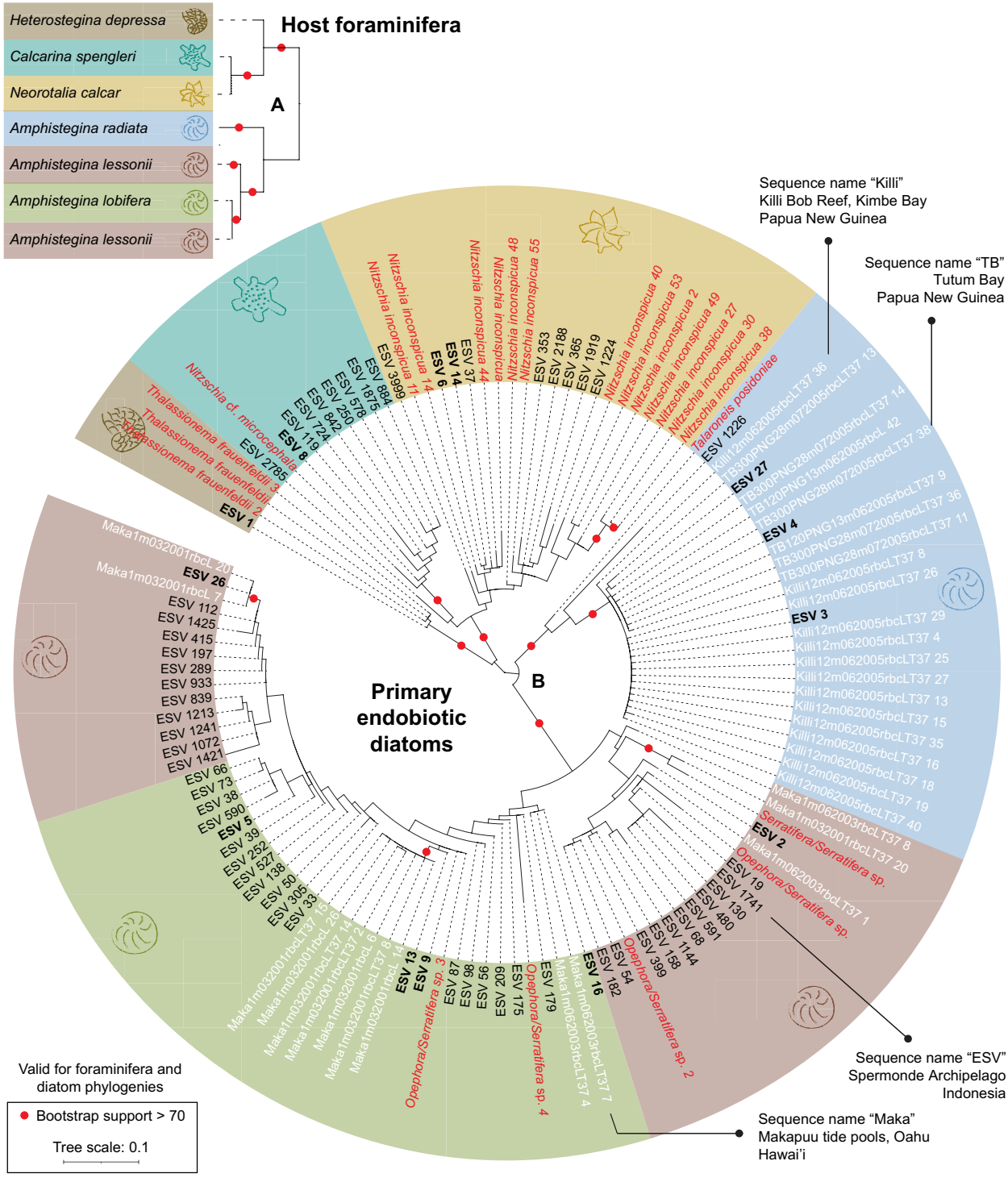

Fig. 4. Overview of the phylogenies of hosts and algal endobionts. Phylogenetic relationships of the host species (**A**) and the primary diatoms (**B**). Molecular distances between all ESVs assigned to the primary diatom per host specimen (black, the most abundant ESV in bold), the reference diatom sequence from the database R-Syst::diatom v8 database (*94*) (red) and the diatom clones of *A. radiata*, *A. lessonii*, and *A. lobifera* samples from Barnes (*44*) (white). The colored regions represent the foram host species in which the diatom is predominantly living. Supporting bootstrap values of >70 are indicated at the branches with a red dot. The tree scale and bootstrap legend are valid for both phylogenies. Note: the phylogenies are restricted to the forams and primary diatom species studied here, and therefore incomplete.

endobiomes and to provide hints toward the acquisition of those endobionts. The diatom and bacterial endobiomes of all foram host species were significantly different from the seawater and the substrate microbiomes (Dunn's pairwise statistical test, table S1). The 20 most abundant diatom and bacterial ESVs in the host species were observed on average in low relative read abundance (<5%) in the environment (Fig. 5, A and B). ESVs observed in the host endobiomes and the environment were more likely to be found in the substrate than in the seawater (Fig. 5, C and D). Nonetheless, 15 diatom ESVs (considering also singletons) were shared between the foram, the seawater, and the substrate samples, of which four ESVs (ESV1, ESV2, ESV3, and ESV4) dominated the diatom endobiome in three foram host species (Fig. 5, A and C).

DISCUSSION

Single-cell metabarcoding of six diatom-bearing LBF species along an environmental gradient revealed a highly specific host-diatom relationship, where each host species harbored a unique diatom dominating at least 90% of the endobiomes. These results were significantly supported by the RDA and the species occupancy modeling. In contrast, the bacterial endobiome was highly variable between

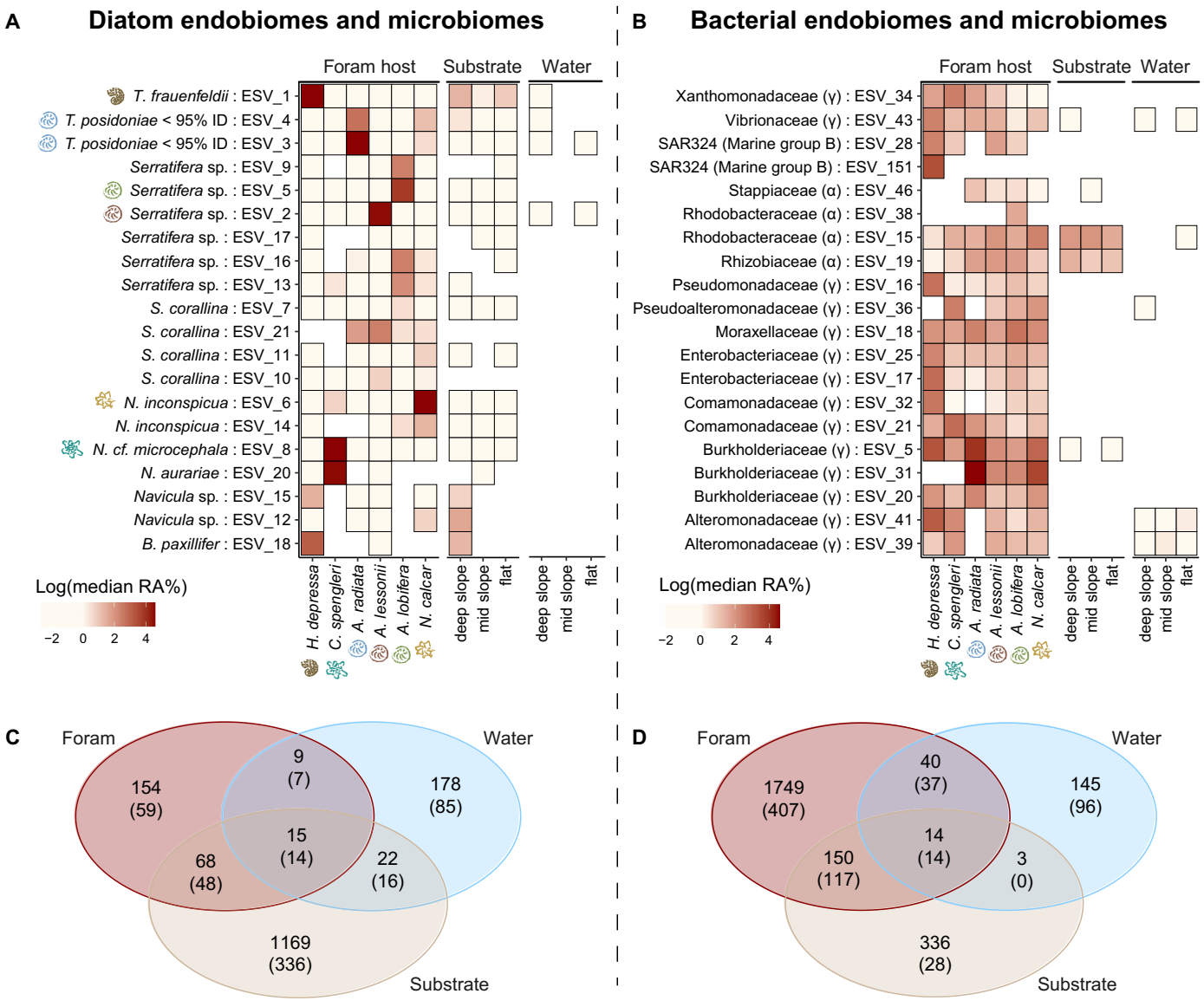

Fig. 5. Prevalence of the endobionts in the environment. Median relative read abundance (RA) of the 20 most abundant diatom ESVs (**A**) and bacteria ESVs (**B**). RA is color coded following a logarithmic scale. Median RA of <1% is indicated by an off-white color and >1% in a gradient of reds. The diatom ESVs were classified at species level, and the bacteria ESVs at the family level (class of Proteobacteria in parentheses). Each host foraminifera species has its own logo, highlighting the most abundant and prevalent ESV in each host. Number of ESVs shared between the forams, seawater, and substrate samples for diatoms (**C**) and for bacteria (**D**), considering all ESVs. The number in parentheses shows the number of shared ESVs considering only ESVs present in at least two samples of the same sample type, where singletons were removed.

host species, with bacterial relative read abundances rarely exceeding 35% in the endobiomes. The 20 most abundant endobiotic diatoms and bacteria in the host were rare, if not absent, in the surrounding environment.

Highly specific host-algae relationship

We consistently observed one primary diatom endobiont species in each host species, congruent with our hypothesis. Previous studies based on culturing isolated microalgae (*26*, *34*, *45*) reported a more flexible composition of the algal endobiome, with more than 10 algal species. Furthermore, direct comparisons between culturing versus cloning and metabarcoding of diatom DNA directly isolated from the intact host demonstrated an even more diverse microalgal community than the culturing experiments (*34*, *44*). Unexpectedly, most of diatom species identified from cultures, often dominated by *Nitzschia* spp., were not the same as the ones identified directly from the host specimens, both histologically and molecularly (*44*). Additionally, we observed little similarity between the environmental microalgal communities and the host's diatom endobiomes, underlining the rarity of the primary diatom endobiont outside the host. Following our results in combination with observations by Barnes (*44*), Prazeres *et al.* (*33*), and Versteegen *et al.* (*42*), we postulate that the primary diatom endobiont identified by histology, cloning, and metabarcoding is the main (mutualistic) endosymbiont and that many microalgae identified from culturing were likely prey or biofilm forming on the shell. Nonetheless, we cannot disregard that most studies cover largely different geographical locations, thus acknowledging that some of the variations observed between isolation techniques might be due to regional differences within morphospecies.

Histological studies have shown that the main endosymbiotic diatoms do not form a frustule inside their host (*46*), which is the main morphological aspect used to identify diatoms in cultures. From this, we infer that either the formation of a frustule is suppressed by the host or the capability of frustule formation might have been lost altogether as an adaptation to an obligate endosymbiotic lifestyle by the diatoms. If the endosymbiotic diatoms do not produce a frustule outside the host, then it is expected that discrepancies arise between the findings from culturing and DNA due to the impossibility of identifying the microalgae [see (*44*)]. Genetic and morphological modifications of microalgae between the free-living and symbiotic stages have been observed in other obligate host-symbiont systems. For example, the phytoplankton *Phaeocystis cordata* is a known symbiont in Radiolaria, yet also ubiquitous in the surface water of the ocean (*47*). The endosymbiotic *P. cordata* was 10-fold larger and had a higher volume of chloroplasts and associated thylakoids (units within chloroplasts) compared to their free-living counterparts (*47*). To conclude, we contribute to resolving the controversy by highlighting that the main endosymbiont is host-specific in diatom-bearing forams.

The rarity of the abundant diatom taxa in the surrounding environment raises the question of how endobionts are acquired. The acquisition of endosymbionts can go two ways: via horizontal transmission, i.e., phagocytosis of organisms from the environment, and vertical transmission, i.e., heritable from parent to offspring (*48*). The high fidelity of the host-diatom relationship resulting from our study suggests that vertical transmission of the endosymbiont is the main acquisition mechanism in diatom-bearing foraminifera. A live recording further showed vertical transfers of algal endosymbionts during asexual reproduction (*29*). Additionally, theories advanced that sexually reproduced LBF juveniles might bear symbiotic cells, a hypothesis based on cell and zygote sizes (*48*). Our results showed that the host species *A. lobifera* had the highest variability in the diatom endobiomes across all host species, by hosting multiple closely-related diatom strains of the genus *Serratifera*. Freshwater diatoms were observed to have high intragenomic variability and bear multiple copies of the *rbcL* gene within a single species (*49*), supporting the presence of a single species of endosymbiotic diatom in *A. lobifera* and its high host specificity. On the other hand, endosymbionts with closely-related strains can exhibit markedly different physiological traits and functional roles, as shown in the thermotolerance of dinoflagellates (*50*–*52*), which may have implications in the resilience of the host to changing environmental conditions. Whether the latter statement is also applicable to diatoms needs further investigation. Compared to all other host species we studied, *A. lobifera* is distributed worldwide (*33*) and known to be an invasive species in the Mediterranean Sea (*53*, *54*). Insights from intragenomic variations revealed that the expansion of *A. lobifera* from the Red Sea toward the Mediterranean Sea was at the cost of its sexual reproduction potential (*54*). As such, vertical transmission of the diatom endosymbiont via asexual reproduction is likely the predominant scenario; however, the reasons behind the higher intragenetic diversity of diatoms in *A. lobifera* remain uncertain.

Our study is limited to six diatom-bearing LBF from the Spermonde Archipelago and is not directly applicable to foraminifera from all other geographical regions or those hosting different microalgal groups. However, phylogenetic analyses from direct single-fragment sequencing demonstrated that chlorophyte-bearing forams harbor chlorophyte species that cluster closely to each other (*55*), suggesting that a similar host specificity of the main symbionts may hold for chlorophyte-bearing LBF. However, dinoflagellate-bearing LBF were shown to harbor multiple genera of Symbiodiniaceae (*56*, *57*), lacking any host-specific patterns (*58*) and influenced by environmental conditions (*59*). Additional studies using single-cell metabarcoding or metagenomes of the hosts are needed to confirm the symbiont specificity and the nature of the algal endobiome in other host groups.

Conserved bacterial metabolic functions across hosts and sites

Contrary to the host specificity of the diatom symbionts, we observed no specific or dominant bacteria in the diatom-bearing forams, suggesting a flexible nature of the bacterial endobiome. In case of an obligate symbiotic association with bacteria, we would have expected a uniform presence of one or more ESVs across most host specimens, which were not shown in our data. The bacterial endobiomes were different between the host species within the same sample and along the gradient of turbidity, but the 20 most abundant ESVs were almost equally prevalent across host species. Although the bacterial endobiome was partially overlapping with the substrate microbiome, bacterial ESVs found in high abundance in the hosts were rare in the environment. Salonen *et al.* (*60*) observed similar results by studying intertidal nonphotosymbiotic small benthic forams, and they suggested that those bacteria were likely not prey. We suggest that bacterial endobionts might rather have a facultative symbiotic relationship with the host or with the endosymbiotic diatom, in both cases likely facilitating the host performance. We observed highly conserved bacterial metabolic functions between all host species, despite the variability and the flexibility of the

bacterial endobiomes. Most of the putative functions of the bacteria concentrate within the nitrogen and sulfur cycles, through ammonia oxidation, nitrite reduction, and sulfate reduction. While those functions were also expressed in the environmental microbiome, our results show unique bacterial communities living within the host foram, different from the environment, suggesting that those functions are beneficial to the host metabolisms. Functions associated with the nitrogen cycle appear to be also important amongst other photosymbiotic organisms like the benthic upside-down jellyfish *Cassiopea xamachana* (*61*), reef invertebrates (*62*), and many coral species (*63*–*66*). Additionally, less dominant functional roles unique to the foram bacterial endobiomes corresponded to chitin and xylan degradation, which are possibly related to food waste and nutrient recycling. Chitin-degrading bacteria were observed to settle on diatom frustules and to degrade it (*67*), an advantageous mechanism to help forams process preyed-upon diatoms to extract most of its nutrient. The efficient nutrient use by the foram holobiont through its mixotrophic lifestyle could be facilitated by the metabolic versatility of various bacterial endosymbionts. Additionally, chitin-degrading bacteria were recognized to participate in the immunity of the host through fungal pathogen defense in plant-microbe interaction (*68*). Whether such an immune mechanism is active in marine protists needs to be further tested in controlled culturing laboratory experiments.

Bacterial endosymbionts might, furthermore, contribute to the supply of inorganic nutrients to photosymbionts, in addition to those supplied by the host. Diatoms lack the ability to fix nitrogen and, therefore, need nitrogen-fixing endosymbionts such as cyanobacteria and some gammaproteobacteria to thrive in case bioavailable forms of nitrogen are limiting (*7*, *69*). Our data show that cyanobacteria of the genus *Synechococcus* and gammaproteobacteria of the genus *Alteromonas* were especially abundant in the host species *A. lobifera*, *A. lessonii*, and *N. calcar*. *Synechococcus* spp. were also observed to participate in nitrogen fixation in reef-building corals (*70*). Furthermore, *Alteromonas* sp. and *Vibrio alginolyticus* were observed to fix nitrogen that was readily incorporated by the algal symbiont in corals (*65*). As a result, host-associated bacteria may also be in direct symbiosis with the microalgal symbiont, which, in turn, benefits the host and improves its adaptability to changing environmental conditions. The bacterial endobiome may, therefore, play a role in filling in nutritive gaps of the foram holobiont, potentially contributing to the host adaptation to a large range of environmental conditions (e.g., increase water temperature and eutrophication) (*40*, *61*). To further investigate the functional diversity of the microbial community in foraminifera and their role in the adaptation of the host to changing environmental conditions, metagenomic and metatranscriptomic analyses might validate our conclusions. Additionally, it would help us identify the biologically relevant interactions between the host, the photosymbionts, and the bacteria, including the roles that the endobiome plays in the host's physiology.

Adaptation to changing conditions: A comparison with the coral holobiont

Algal-bearing LBF often live side by side to the well-studied reef-building corals. Similarly to forams, those marine mixotrophs are complex holobionts composed of a variety of eukaryotic and prokaryotic partners in a coelenterate host (*71*). Corals form an obligatory symbiosis with photosynthetic dinoflagellates (*72*). Their bacterial endobiome provides nutrients to the host and its endosymbiotic dinoflagellates (*27*, *71*). Corals are known to be specific in the choice of dinoflagellates. One coral species usually hosts one genus of the family Symbiodiniaceae (*73*) as the dominant symbiotic group while hosting some other genera at lower abundance (*74*–*76*), similarly to the results in diatom-bearing forams in our study. However, some species of corals host two or more genera of the family Symbiodiniaceae and were observed to shift, at least temporarily, the relative abundance of those genera with shifting environments (*74*, *77*, *78*). This flexibility was suggested to align with a shift in the host metabolic requirements (*79*) and correlates with the host geographic range (*80*). However, the ability of some corals to shift or adapt their symbiotic community is species specific and context dependent (*74*, *81*). Environmental conditions, such as sea surface temperature, pH, salinity, and photosynthetically active radiation, can also shape the Symbiodiniaceae community within the coral holobiont (*74*). Equivalent changes in the endosymbiont communities were not observed in the six species of diatom-bearing LBF from the Spermonde Archipelago. Contrastingly, we rather observed a species-specific relationship between the six host foram and their respective endosymbiotic diatom. This species specificity held even across geographical distances in *Amphistegina* spp. (Papua New Guinea and Hawai'i) (*44*). Three of the six host species (*A. lobifera*, *A. radiata*, and *N. calcar*) show significant variations in their diatom endobiomes along the regional gradient of turbidity in the Spermonde Archipelago. These variations are likely associated with the diatom endobionts present at low background abundance. Our results also show that some foram specimens (6 to 33% of each host species) hosted a different symbiotic species of diatoms from the prevalent primary diatom species. These different foram-diatom associations did not correlate with a change in (a)biotic factors, unlike the patterns observed in coral-Symbiodiniaceae associations (*74*). In that sense, diatom-bearing LBF appear less flexible than reef-building corals regarding their host-algae symbiosis, but additional studies across a wider range of environmental conditions and geographical localities need to be conducted to confirm this hypothesis.

Our sampling design covered multiple depths and varying levels of light intensity, associated with turbidity, and eutrophication (*82*) within a single region. We observed clear variations in the bacterial endobiomes, which was significantly shaped by the microhabitat, following changes along depth and the turbidity gradients. We, therefore, speculate that the different environmental conditions triggered the dynamism observed in bacterial communities. Similar shifts in the bacterial endobiome in photosymbiotic organisms were also recently highlighted through bleaching events (*40*), across water depths (*42*), and the reef shelf (*39*). The flexibility of the bacterial endobiome appears to not affect the basic bacterial metabolic functions performed within the foram host, with different but functionally equivalent taxa, potentially contributing to the host adaptation and proliferation in different environmental settings. Corals also have a highly flexible bacterial microbiome primarily dependent on abiotic factors associated with the microhabitat (*83*, *84*). Despite their eukaryotic and prokaryotic flexibility, reef-building corals are sensitive to environmental fluctuations such as heat waves (*85*, *86*). A study contradicting the paradigm that generalists are more resilient to change found that flexibility in the algal symbiosis correlates negatively with resilience (*87*). Putnam *et al.* (*87*) analyzed the dinoflagellate endobiomes of *Acropora*, *Pocillopora* (both generalists), and *Porites* (specialist) from Moorea and observed that generalist

species had flexible host-algae relationships while being less resilient to sudden changes in the environmental conditions compared to their specialist neighbors. Yet, a contradicting study shows that the renewal of the photosymbiotic partners during a heat wave with heat-tolerant symbionts allowed the host to tolerate the prolonged exposure to thermal stress (*88*). This flexibility was also observed in LBF, both within their microalgal (*34*) and bacterial endobiomes (*40*). How the activities of the bacterial endobiome and its flexibility benefit (or hinder) the adaptation of marine mixotrophs to environmental stress remains largely unanswered. Nonetheless, the flexible nature of those holobionts, lying within its prokaryotic endobionts, appears to be an advantage to environmental adaptation.

MATERIALS AND METHODS

Experimental design

In August 2022, benthic samples ($n = 16$) containing forams (unicellular organisms) were collected from six reefs located at different islands (Samalona, Kodingareng Keke, Pajenekang, Badi, Langkai, and Kapoposang) in the Spermonde Archipelago, South Sulawesi, Indonesia (see Fig. 1). At each reef, we visited three sites: the reef flat (~1-m water depth), the mid-reef slope (~10 m) and the bottom of the reef slope (~20 m) (see table S2 for details). Forams were collected by filling a cotton bag (18 cm by 32 cm) with coral rubble, sand, and/or algae. The coral rubble and the algae were brushed on the boat directly after collection to detach the forams inhabiting the surface. The brushed product concentrated in forams was immediately transferred to an 8-ml tube filled with ethanol 96% on the boat and preserved at −20°C on shore until brought to Naturalis Biodiversity Center (NBC), Leiden, The Netherlands, for analysis. We thank the Indonesian authorities (Ministry of Law and Human Rights, Republic of Indonesia) for approving the collection of reef sediment samples (permit holder, E. Girard; permit number 2C11FB0145-W).

Data on free-living bacterial and diatom communities in the immediate environment (substrate and seawater from the benthic boundary layer) were collected at the exact same location as our foraminifera samples (SRA BioProject PRJNA1105779), published by Girard *et al.* (*82*). In summary, the substrate was collected in falcon tubes (coral rubble, sand, and/or algae), and the seawater was exchange with ethanol 96% on the boat immediately after collection. Seawater was collected in triplicates with 50-ml syringes, which were stored in a cool box on the boat until filtered through a single-use 0.2-µm filter on shore. The filters were subsequently stored in a tissue lysis buffer (ATL) at room temperature until DNA extraction was performed at NBC. Extracted DNA from all the samples were amplified with the same primer sets for bacteria and diatoms as for the foraminifera samples described in our study. We used these data to compare the microbiome in foraminifera with its counterpart from the environment (table S2).

Morphological and molecular identification of foraminifera

LBF specimens ($n = 254$) were isolated from the brushed product with soft tweezers under a stereo microscope. Specimens were selected on the basis of their color, indicating the presence of photosymbionts, to distinguish between live and dead specimens. Between four and seven specimens identified as *A. lessonii* ($n = 75$), *A. lobifera* ($n = 36$), *A. radiata* ($n = 49$), *C. spengleri* ($n = 10$), *H. depressa* ($n = 55$), and *N. calcar* ($n = 30$) were selected from each sampling site (Fig. 1). Note that *C. spengleri* was only found at two sampling sites, explaining its limited number of specimens to 10. Species identification was based on morphological features of their tests, following the criteria outlined by Renema (*89*). These particular species were chosen because they are all known to be diatom-bearing, abundant in the samples, as well not having spikes on their test, which reduces the risk of contamination of material adhering to the test (see below). Nonetheless, we acknowledge that the porosity of certain tests might increase the potential for contamination, which is why we used stringent filtering and postprocessing of the datasets. Pictures were taken of every specimen to verify the identification after DNA extraction. Each specimen was carefully stored in separate tubes with ethanol 96% to prevent any potential cross-contamination.

Before DNA extraction, the specimens underwent multiple cleaning steps to thoroughly eliminate external microorganisms and organic matter from the shells. First, specimens were brushed with a thin brush and transferred to a new individual tube containing clean ethanol 96%. The utensils used in this step were sterilized between the cleaning of each individual. Then, the specimens were rinsed three times with Milli-Q water, with vortexing carried out between each rinse to enhance the removal of remaining impurities. Last, the cleaned specimens were stored in a new tube filled with ethanol 96%. Each specimen was dried in a new 1.5-ml Eppendorf tube and crushed using a sterile metal pestle. We performed the DNA extraction and amplification of the cytochrome c oxidase subunit I (COI) mitochondrial region (*90*) following the protocol of Girard *et al.* (*91*), using 35 cycles instead of 40 in the polymerase chain reaction (PCR) program. During DNA extraction, all manipulations were performed at a clean bench to enact a sterile environment. To serve as negative controls, the same method was carried out in tubes without any biological samples. Additionally, a negative control was performed with Milli-Q water instead of DNA template for each PCR run to check for potential (cross-)contamination. The samples were sequenced using Sanger sequencing at BaseClear B.V., Leiden, The Netherlands. We processed the sequences in Geneious Prime (version 2023.2.1). The sequences were assembled with de novo assembly, checked, and edited according to the quality of the base and cropped to the same length. The sequences were assigned a taxonomic name with BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). If the closest hit corresponded to the morphological species identification, then we confirmed the taxonomy of the foraminifera host (see table S2); otherwise, the sample was disregarded ($n = 4$).

Library preparation for metabarcoding of the endobiome

To study the endobiome of foraminifera, we performed single-cell metabarcoding on individual unicellular foraminifera specimens, which host bacterial and diatom cells. Both the bacterial (variable regions V4 and V5 of the 16*S* rRNA gene) and the diatom [variable region ribulose-bisphosphate carboxylase (*rbcL*) gene] communities were sequenced from the extracted DNA in the previous step. The two libraries were prepared from a two-step PCR to first amplify the target region and, second, to label the DNA using IDT 10–base pair unique dual indexes (Integrated DNA Technologies, Coralville, IA, USA). We used the same primers and the library preparation method as published by Girard *et al.* (*82*).

To ensure even representation during sequencing, all samples were pooled in equimolar ratios using the Qiagility robotic workstation (QIAGEN) into a subpool. The DNA concentration of the subpools was measured on the 4150 TapeStation System (Agilent) and equimolarly pooled into one final pool, one per marker. Last, the

samples were sequenced on an Illumina NovaSeq 6000 platform (250 paired-end reads) at BaseClear B.V. (Leiden, The Netherlands).

Metabarcoding data processing

Demultiplexed reads for both bacteria and diatoms were processed separately in APSCALE (Advanced Pipeline for Simple yet Comprehensive AnaLysEs), a VSEARCH-based software to correct amplicon errors and identify chimaeras (*92*, *93*). The details on the processing, settings, and programs used, as well as the sequence treatment report are compiled in tables S3 and S4. The sequences were denoised into ESVs. The processing pipeline resulted in an ESV table, free of chimaeras. To be conservative as well as to limit cross-contamination and index-hopping, we removed the ESVs with less than 0.1% relative abundance per sample, and we analyzed samples with a total read number > 1000 reads. We merged the triplicates (the reads were summed) and analyzed ESVs present in at least two of the triplicates. Last, reads were normalized to the sample with the lowest number of reads (diatoms of 5927 reads and bacteria of 1130 reads). The ESVs were identified with Megablast (80% minimum coverage and 80% minimum similarity) using Silva 138.1 SSUParc database for bacteria (www.arb-silva.de/documentation/release-1381/) and R-Syst::diatom v8 database (2020) for diatoms (*94*). The sequence percentage identity threshold (ID) used for bacteria followed the study of Yarza *et al.* (*95*). Because we are only interested in bacteria, ESVs assigned to mitochondria, chloroplasts, and Archaea were disregarded. Diatom ESVs (strictly classifying within the phylum Bacillariophyta) were considered to be a diatom species if the percentage ID was >95% similar to the reference, a threshold commonly used for diatoms (*96*, *97*). To verify this threshold, we calculated a similarity distance matrix in Geneious Prime (version 2023.2.1), using the aligned *rbcL* barcode from the entire R-Syst::diatom v8 database (fig. S7).

Quality control of the dataset

We identified and removed potential ESV contaminants using the R package "decontam" (*98*). We compared the samples with the negative controls using the function isContaminant() with the prevalence threshold set at 0.2. Once a contaminant was identified, its read number was set to zero in the corresponding samples. To strengthen the removal of contaminants in the bacteria dataset, we manually screened through ESV assignment results and further removed ESVs which are documented to be associated with the human microbiome ("human gut metagenome," "human oral metagenome," "human metagenome," "*Streptococcus*," "*Cutibacterium acnes*," and "*Burkholderia pseudomultivorans*"). We performed the latter steps to be conservative in our analysis (*99*) to overcome possible method-related (cross-)contamination and index-hopping, ensuring the study of relevant taxa only.

Statistical analysis

After normalizing the number of reads, the data were transformed into ESV relative abundances (%) per sample. The following analyses of the data test for the host specificity of the endobiomes and aim to identify the main drivers shaping the two endobiomes in the hosts. We hypothesize that symbiotic diatoms are host specific, with one species dominating the community while being scarce in the environment, whereas the bacterial community within the host is more flexible and reflects the composition of the environmental microbiome. To confirm the different specificity levels of the endobiomes, we assessed the presence of the endobiomes in the environment by analyzing the proportion of ESVs shared between the host and the surrounding environment. Additionally, we identified potential symbionts within the endobiomes, which supports the concept of specificity. We performed all data manipulations and analyses in R version 4.4.0 (*100*). Mean relative abundances exclude zeros, and the SD is displayed whenever possible.

Determining the host-specificity level of endobiomes

To understand which variables have the greatest impact on the endobiomes, we performed an RDA on all ESVs remaining after quality control with the function rda(…, dist = "bray", scaling = "species"), using the Bray-Curtis distance matrix, from the R package "vegan" (*101*). The analysis was performed for both bacteria and diatom endobiomes. Different groupings were tested: host species (*A. lobifera*, *A. lessonii*, *A. radiata*, *C. spengleri*, *H. depressa*, and *N. calcar*) and the local environmental variables around each sampling site (referred to as "microhabitat"), such as substrate type (seawater, coral rubble and algae, coral rubble and sand, and coral rubble), water depth (1, 10, and 20 m) and island (Samalona, Kodingareng Keke, Pajenekang, Badi, Langkai, and Kapoposang). We performed an ANOVA to test the significance of the RDA model, including testing the significance of the axis (by = "axis" with 500 permutations) and the environmental parameters (by = "terms" with 200 permutations). Additionally, we performed the RDA and ANOVA analyses on each foram host species separately. Subsequently, we performed the post hoc Dunn's statistical test to verify pairwise similarities and dissimilarities between sample types, using the function dunn_test() from the R package "rstatix" (*102*). The Dunn's test was performed on both the ESV presence-absence dataset and the ESV relative abundance, because the communities from the substrate, the seawater, and the foraminifera were extracted with different extraction kits, which can create a bias in the relative abundance (*103*).

Community composition of endobiomes

We hypothesized a skewed distribution of the diatom community with a dominating species and a more evenly distributed bacterial endobiome. To test this hypothesis, we calculated multiple diversity indices [Pielou evenness, Simpson's index (model 1-D), Shannon-Wiener index, and species richness] using the function diversity() from the R package "vegan." Additionally, we displayed the community composition in the form of bar charts and heatmaps using the 20 most relatively abundant ESVs of the endobiomes. These ESVs were identified by summing the relative abundance of every ESV across all foram hosts.

Relationship between the endobionts and the host

Two additional analyses were performed, where only the ESVs assigned to the most abundant diatom species endobionts in every host foraminifera were selected. First, we tested whether the most abundant diatom endobionts were more likely to be found in a specific host foraminifera using an occupancy model from the R package "camtrapR" (*104*). The model uses Markov chain Monte Carlo to assess the fit and takes the environmental community as a baseline for comparison. Last, we performed a phylogenetic analysis to compare the foraminifera phylogeny with that of the diatom ESVs assigned to the primary diatom endobiont, their closest hits on the R-Syst::diatom V8 database and the diatom clones from *A. lessonii*, *A. lobifera*, and *A. radiata* from Barnes (*44*), representing some of the few available rbcL sequences from foraminiferal endosymbionts in the literature. The sequences were aligned in Geneious Prime using MAFFT (*105*) with default settings and then trimmed to the length of the *rbcL* amplicons, and duplicate sequences were removed. We

built the phylogenetic tree with PhyML (*106*) with the option substitution model HKY85 (*107*) and 100 bootstraps. The same method was applied on the Sanger foraminifera COI sequences for the foraminifera phylogeny.

Taxonomy-based functional prediction

To infer functional roles of bacteria in the foram holobiont, we used the statistical tool for comparative metagenomics METAGENassist (*108*). The software predicts a range of phenotypes based on taxonomy. Following the authors' protocol for data input, we compiled two csv-format files: one with the relative abundance of every taxon and one with the metadata. The taxa table was built by merging the number of reads (sum) per taxon for a foram host species at a certain depth and certain island, therefore grouping biological replicates to obtain an overview of the functions across all habitats. The metadata file included the following variables: foram host species, island, depth category, and substrate type. The data were filtered on the basis of the interquantile range to improve the robustness of the model [number of ESV removed during filtering = 516 (40%)]. Because the dataset was normalized during the data analysis (to the lowest number of reads of all samples), no additional normalization was performed.

Supplementary Materials

The PDF file includes:

Figs. S1 to S7

Legends for tables S1 to S4

Other Supplementary Material for this manuscript includes the following:

Tables S1 to S4

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