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A novel menthol-DCMU bleaching method for foraminifera: generating aposymbiotic hosts for symbiosis research

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Abstract
Understanding the cellular symbiosis between marine holobionts and their endosymbiotic algae is crucial for predicting the resilience of coral reefs to climate change. While bleaching protocols have been widely applied to model organisms such as corals and sea anemones, their application to other symbiotic taxa remains underexplored. This study presents the first application of a menthol-DCMU bleaching method on larger benthic foraminifera (LBF), important calcium carbonate producers in reef ecosystems. Two species, *Amphistegina lobifera* (harbouring endosymbiotic diatoms) and *Sorites orbiculus* (harbouring Symbiodiniaceae dinoflagellates), were tested for their suitability for controlled symbiont removal. The study aimed to establish a non-lethal and effective bleaching protocol to generate aposymbiotic hosts for experimental symbiosis research. In a two-step approach, we first determined an optimal menthol concentration (0.19 mmol l^{-1}) and then assessed its effect on growth, motility (as a fitness indicator), and mortality over four weeks. The treatment successfully induced an aposymbiotic state in 100% of *A. lobifera* specimens with minimal effects on motility and mortality. Growth was inhibited in both species, preventing calcite deposition during the bleaching process. This study establishes menthol-DCMU bleaching as a viable method for producing aposymbiotic foraminifera, allowing controlled studies of symbiont uptake, symbiosis establishment, and host-symbiont specificity in this important taxon.

Keywords Foraminifera · Bleaching · Coral reefs · Symbiont manipulation · Symbiosis · Symbiodiniaceae · Endosymbiosis

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1 Introduction

Foraminifera are major contributors to global ocean carbonate production, with both benthic (seafloor-dwelling) and planktic (open-ocean) species playing significant roles. Collectively, they produce approximately 1.4 billion tonnes of calcium carbonate annually, accounting for nearly 25% of the total carbonate production in the world's oceans (Langer 2008). Reef-associated foraminifera, also called LBF (Large benthic foraminifera), contribute ~43 million tonnes annually (Langer et al. 1997). They rely on photosynthetic endosymbionts, such as diatoms and dinoflagellates, for their growth and calcification (Lee 2006, Prazeres et al. 2021), similar to corals and are associated with a wide variety of Symbiodiniaceae (Pochon et al. 2004; Fay et al. 2009; LaJeunesse et al. 2018). However symbiont diversity extends beyond these groups to include chlorophytes and rhodophytes (Leutenegger 1983; Lee 1998). While they form stable and persistent symbioses with eukaryotic

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partners, their associations with prokaryotic microbes are flexible and site-specific (Prazeres et al. 2017).

The persistent endosymbiosis with eukaryotic partners is ecologically significant, as coral reef holobionts enhance their calcification and growth in oligotrophic tropical seas through this symbiosis (Muscatine and Porter 1977; Morris et al. 2019). When this symbiosis is disrupted by local or global anthropogenic stressors, coral reefs decline (Hoegh-Guldberg 2004; Wild et al. 2011). In contrast to other coral reef holobionts, LBF have been much less studied compared to the well-documented symbioses in cnidarians or corals. To conserve biodiversity, it is crucial to better understand eukaryotic partnerships in non-coral holobionts and to develop multi-species strategies to help natural populations adapt to climate change. This emerging field, known as "assisted evolution", is being explored as a potential strategy to prevent further coral reef decline (van Oppen et al. 2015). Experiments include the synthesis of new and more thermally robust host-symbiont pairings through experimental evolution of the symbionts (Scharfenstein et al. 2024). In line with these efforts, our work provides a methodological approach for generating aposymbiotic LBF, using menthol-DCMU as bleaching agents, with the ultimate goal of addressing new scientific questions on foraminifera, and perhaps enhancing the thermal tolerance of LBF. This methodological approach could be particularly relevant, as LBF naturally associate with a wide diversity of dinoflagellate symbionts (Pochon et al. 2004; Fay et al. 2009) and diatom symbionts (Lee 2006, Prazeres et al. 2021), which may offer opportunities for exploring thermal tolerance enhancement.

Bleaching protocols have been improved over the years, becoming more effective and faster. In the past, a combination of heat or cold stress, dark incubation, and/or treatment with the photosynthetic inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), has been used to induce photosymbiont loss, especially in cnidarians (Belda-Baillie et al. 2002; Xiang et al. 2013; Lehnert et al. 2014). However, this approach is time consuming, often taking several months to produce fully aposymbiotic specimens. In recent years, a faster and more effective method has been developed over the past decade that induces bleaching of corals and sea anemones through a combined incubation with menthol and DCMU (Wang et al. 2012; Matthews et al. 2016; Puntin et al. 2023). Menthol blocks voltage-operated sodium channels, causing local anaesthetic effects (Haeseler et al. 2002). The exact biochemical reasons why it induces bleaching in cnidarians are poorly understood, but it has little or no discernible effect on the overall condition of the host (Wang et al. 2012; Matthews et al. 2016).

The use of model systems in coral reef research has accelerated in recent years, with, for example, the sea anemone *Exaiptasia diaphana* (commonly referred to as "Aiptasia") and the upside-down jellyfish *Cassiopea xamachana* helping to elucidate the processes underlying the cnidarian-dinoflagellate symbiosis (e.g. Weis et al. 2008; Medina et al. 2021). Additionally, the menthol bleaching method has been applied to a scleractinian coral model, *Galaxea fascicularis* (Puntin et al. 2023). Currently, we lack an equivalent model system and a menthol bleaching method for LBF, despite their significant contributions to coral reef productivity and biogeochemical cycling (reviewed in Narayan et al. 2022). Larger benthic foraminifera are easy to collect for highly replicated experiments, making them ideal candidates for a new model system. In particular, the foraminifer *Amphistegina lobifera* is a tropical to subtropical species (Langer and Hottinger 2000) for which a recent cultivation protocol allowed its growth and reproduction in artificial seawater (ASW) with minimal feeding of marine microalgae (Schmidt et al. 2015, 2016). Collection of samples has also become easier, since the opening of the Suez Canal 150 years ago, when Amphisteginids, Soritids, and other LBF have invaded from the Red Sea and reached the Mediterranean (Zenetos et al. 2008; Weinmann et al. 2013). As the Mediterranean Sea warms, several foraminiferan species are expanding their geographic thermal tolerance range westward (Guastella et al. 2019). For Soritids, the population in northern Israel is known to be genetically identical to that in northern Red Sea (Merkado et al. 2013).

In this study, we tested menthol-DCMU bleaching on two species of LBF, *Amphistegina lobifera* (hosting diatoms) and *Sorites orbiculus* (hosting dinoflagellates). By rearing aposymbiotic LBF, we aim to to inoculate them with different endosymbiotic microalgae, to answer questions about shifts in the symbiont composition in relation to environmental change ('shuffling') and host-symbiont specificity.

2 Materials and methods

2.1 Sampling and culturing of the foraminifera

Samples for this study were collected from a shallow littoral habitat at Capo Passero, Sicily, Italy, Mediterranean Sea (GPS 36.686667, 15.138278), a site known to represent, the 'invasion front' (Raposo et al. 2023). A second foraminiferal species, *Sorites orbiculus*, was obtained from the Red Sea, near the Inter-university Institute for Marine Sciences in Eilat, Israel (GPS 29.501866, 34.917488). Samples were collected by snorkelling at shallow depth (<3 m) by removing small pieces of seabed rubble. On shore, the rubble was brushed to remove sediment and attached foraminifera. Samples were shipped to New Zealand in an insulated container containing filtered natural seawater from their source site in Falcon tubes (Volume: 60 ml). Upon arrival, they

were transferred to freshly prepared artificial seawater (Red Sea Salt) at 37–40 PSU and maintained in a temperature-controlled room at 23–25 °C under white fluorescent light (AQUA-GLO T8 fluorescent bulbs) at 15 µmol photons m^{-2} s^{-1} on a 12:12 h light:dark cycle. Cultures were covered with parafilm to limit evaporation and aerated with a small air pump. For subsequent experiments, they were transferred to local natural seawater (0.22 µm filtered, source: Victoria University of Wellington, New Zealand). A total of three experiments were carried out, which are described in detail below.

2.2 6-week menthol concentration comparison (MCC) & 33-day high concentration trial (HCT)

The Menthol Concentration Comparison (MCC) experiment was designed to find a non-lethal dose of menthol to produce aposymbiotic foraminifera. Four different menthol concentrations in the lower spectrum were used: 0.05, 0.1, 0.19, and 0.25 mmol l^{-1}, using a small sample size ($n=3$) *per* concentration. This Menthol Concentration Comparison (MCC) experiment was carried out on both species using individual foraminifera in their experimental jars, monitoring their response with respect to mortality, motility, and bleaching, using epifluorescence microscopy, over a 6-week time frame. They were kept separate to better observe individual responses and to track individuals using epifluorescence microscopy. In addition, a High Concentration Trial (HCT) was conducted over 33-day time frame exposing *A. lobifera* to higher menthol concentrations (0.35, 0.4, 0.45, 0.50, 0.55 and 0.6 mmol l^{-1}). This experiment was carried out to test whether shock responses to the menthol-DCMU treatment occurred at higher concentrations in this LBF species.

2.3 4-week menthol-bleaching ecophysiology assessment (MEA) measuring growth and mortality

The design of the Menthol Bleaching Ecophysiology Assessment (MEA) was based on the results of the MCC experiment and menthol bleaching protocol developed by Matthews et al. (2016) for rearing aposymbiotic Aiptasia. The final incubation solution used is the same as that used in this study, and consists of 0.19 mmol l^{-1} menthol (Sigma Aldrich, NZ) and 5 µmol l^{-1} DCMU (from a stock solution of 100 mmol l^{-1} dissolved in EtOH; Sigma Aldrich, NZ) in 1 µm-FSW. DCMU acts here as a photosynthetic inhibitor and was added to prevent algal growth and to limit the potential for expelled symbionts to re-enter the host. Foraminifera were incubated in a menthol-DCMU-FSW solution for 6–8 h for five days a week, which was replaced by a FSW-DCMU solution (5 µmol l^{-1} DCMU in 1 µm FSW) for the remaining hours of the day. This 24 h bleaching cycle was repeated on weekdays (5 days per week instead of 4 days per week in Matthews et al. 2016) for a total of four consecutive weeks, while samples were kept in the DCMU-FSW treatment solution (5 µmol l^{-1} DCMU in 1 µm-FSW) during weekends. Epifluorescence microscopy was used to assess bleaching and pseudopod movement at initial and final time points, and growth was also measured, as described below.

2.4 Experimental design to test menthol bleaching success

Foraminifera were kept in screw-capped plastic jars (120 ml) with translucent plastic lids. The jars were placed on a plastic grid illuminated at 30 µmol photons m^{-2} s^{-1} on a 12:12 h light cycle under a fluorescent aquarium light source (AQUA-GLO T8 fluorescent bulbs). Temperature was controlled by a water bath set at 25 °C (Julabo V8 thermal pump). Feeding was performed daily in the evening, immediatly after a water change to the DCMU-FSW solution. A total of 30 µl *Nannochloropsis* feeding concentrate, prepared as described in previous culture experiments (Schmidt et al. 2015), was added per jar.

Each jar contained 50 ml of menthol-DCMU solution in FSW in different concentrations (see above) or FSW only (controls). In the MCC experiment and the HCT, a single specimen of foraminiferal sample was kept in each jar, to allow re-identification of each sample during microscopy and motility assessments. In the MEA experiment, the number of samples per jar was increased to allow statistical analysis and varied from 3 to 16, with the smallest sample size attributed for microscopy and the largest for growth assessments.

2.5 Epifluorescence microscopy

Individual foraminifera from each treatment were visually examined in standard 6-well cell culture plates filled with fresh 1 µm FSW. *Amphistegina lobifera* was imaged at × 10 magnification, and *S. orbiculus* was imaged at both × 4 (to take an overview picture) and × 20 magnification (close-up). The different magnifications were chosen to best observe the reduction in symbiont density throughout the experiment, using an epifluorescence microscope equipped with an attached camera (Olympus BX63, DP73 Camera). Observations were made at initial and final time points of the MEA experiment and weekly for the MCC experiment. High-resolution fluorescence images (2400 × 1800 pixels) were acquired using a DAPI filter (excitation 382–393 nm, 417–477 nm emission), illuminating the symbionts in bright red and the calcium carbonate test in blue. The combination

of epifluorescence and the brightfield setting allowed simultaneous imaging of the symbionts and pseudopodia. During the final image acquisition, additional videos of the pseudopods were filmed to provide further insight into the physiological effects of menthol treatment and to demonstrate pseudopod movement in fully bleached foraminifera.

2.6 Motility and pseudopodial movement

Motility is used as an indicator of holobiont fitness in foraminifera (Schmidt et al. 2011). The experimental design in this study allowed motility to be recorded for A. lobifera as follows: before each water change, the position of each *A. lobifera* specimen was classified, as either a) on the wall (vertical) or b) on the bottom on the bottom of the culture jar (horizontal). If foraminifera we at the bottom of the culture jar, they were regarded as being not active. However, slight movement at the bottom of the jar could not be ruled out as well. The movement in the vertical position is characteristic for this species. The data were used to calculate the percentage motility *per* jar and *per* treatment. Cultures were examined daily on weekdays in both experiments. Motility of *S. orbiculus* in the same experiments was not measured, as this species was not as motile overall, and did not climb the walls of the culturing jars regularly.

2.7 Growth and mortality

For growth measurements, the initial and final sizes of each specimen were imaged using a compact stereomicroscope (Zeiss Stemi 305 with Zen software). From these two measurements, the maximum diameter of each specimen was measured (ImageJ software, Schneider et al. 2012). To obtain growth rates *per* individual, the specimens *per* jar were ordered by size (from smallest to largest individual) and the data were ordered to calculate individual growth rates using the formula in Schmidt et al. (2011).

Mortality of living foraminifera is typically assessed by recording vital cytoplasm colour (Bernhard 2000). In this study, however, fully bleached foraminifera often lacked cytoplasm colour and we used a combination of methods to assess mortality, such as lack of motility, cytoplasm colour and extended pseudopodial nets. Mortality was systematically assessed using the paraments above in the MEA 4-week experiment. It was not systematically assessed after these parameters in the MCC 6-week experiment and the HCT, because here motility was observed to investigate the effect of the bleaching treatment on fitness of the holobiont. After the MEA 4-week experiment a few individuals of *S. orbiculus* were overgrown by algae, which in this case were regarded dead. This may have led to a slight increase in the mortality rate of this species compared to *A. lobifera.*

2.8 Statistics

Statistical analyses and graphs were performed in RStudio, using R version 4.3.0 and the tidyverse and ggprism packages (R Core Team 2023). For growth data in the MEA experiment, the non-parametric Mann-Whitney U test was used to test for differences between treatments (menthol vs. control), as the data were not normally distributed and did not meet the assumption of homogeneity of variances. To analyse the motility data in the 4-week MEA experiment, we used a generalised linear model (GLM) for binomially distributed data with a logit link function, combined with a generalised estimating equation (GEE) approach to account for the temporal correlation between repeated measurements on the same individuals. Specifically, we used the geeglm function within the geepack R package to implement the GEE model. To account for temporal correlation, we included a first-order autoregressive correlation structure (AR-1) in the model. The predictor variables in our model included 'treatment' and 'day of experiment', including the interaction term. The Wald statistic was used to assess differences between model parameters. The Motility of the 6-week MCC (Menthol Concentration Comparison) experiment and the HCT (High Concentration Trial) was not statistically evaluated because of the small sample size ($n=3$).

3 Results & discussion

3.1 6-week menthol concentration comparison (MCC) & 33-day high concentration trial (HCT)

Visual comparison of epifluorescence microscopy images between the different menthol concentrations used for *A. lobifera* (Fig. 1) and *S. orbiculus* (Fig. 2), showed that menthol bleaching was effective. For *A. lobifera*, the visually strongest reduction occurred between days 16 and 33 (Fig. 1), and bleaching also occurred at the lower menthol concentrations. For *S. orbiculus*, there were individual differences between samples (see specimen #2 was not fully aposymbiotic, Fig. 2, conc. 0.25 mmol l^{-1}). However, most samples visually lost their symbionts within the 6-week timeframe (Figs. 1 and 2). Motility, the climbing of *A. lobifera* up the vertical walls of the jar from a flat surface, was additionally assessed to observe the holobiont's response to the menthol stressors. The motility data showed no clear trend for any of the treatments (Fig. 3), therefore the third highest dose of 0.19 mmol l^{-1} was considered safe for the foraminifera (Fig. 3). This concentration has been used successfully in the cnidarian model Aiptasia (Matthews et al. 2016). Therefore, we decided to use the dose of 0.19 mmol l^{-1}, for the 4-week MEA experiment in order to

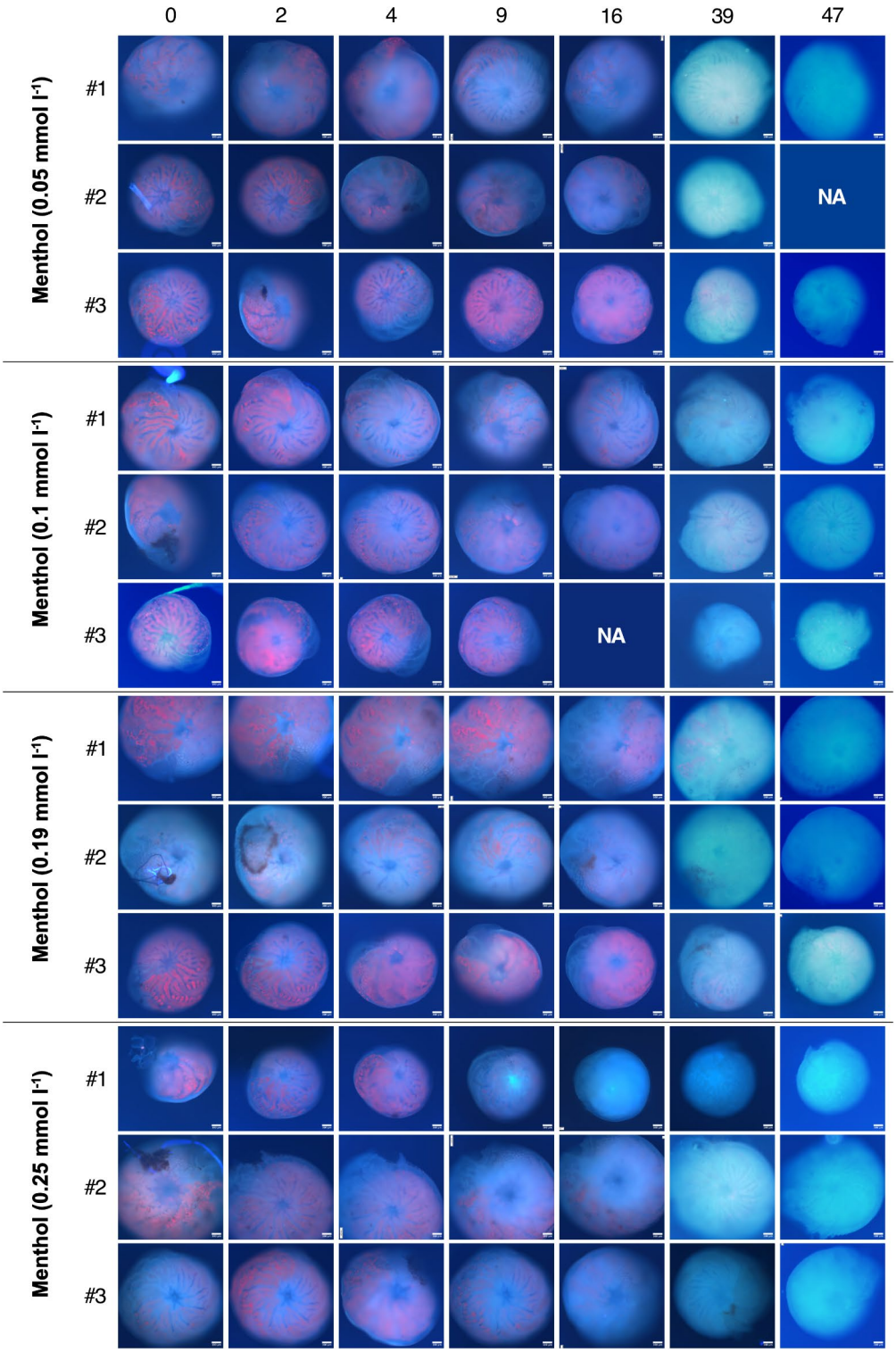

Fig. 1 Epifluorescence microscopy images of all *Amphistegina lobifera* individuals during the 6-week MCC (Menthol Concentration Comparison) experiment at seven different time points (days) and different menthol concentrations (mmol l^{-1}) (superscript^{-1}) . Scale bars are 100 µm.

access the influence of the menthol also on growth rate in a larger sample size.

The HCT High Concentration Trial (up to 0.6 mmol l^{-1}) was performed on *A. lobifera* because it had been previously tested on *Aiptasia*, and caused 50% mortality in this anemone. During the experiment we did not systematically assess mortality, but did assess the fitness of the holobiont by assessing motility (Fig. 4). At the end of the 33-day

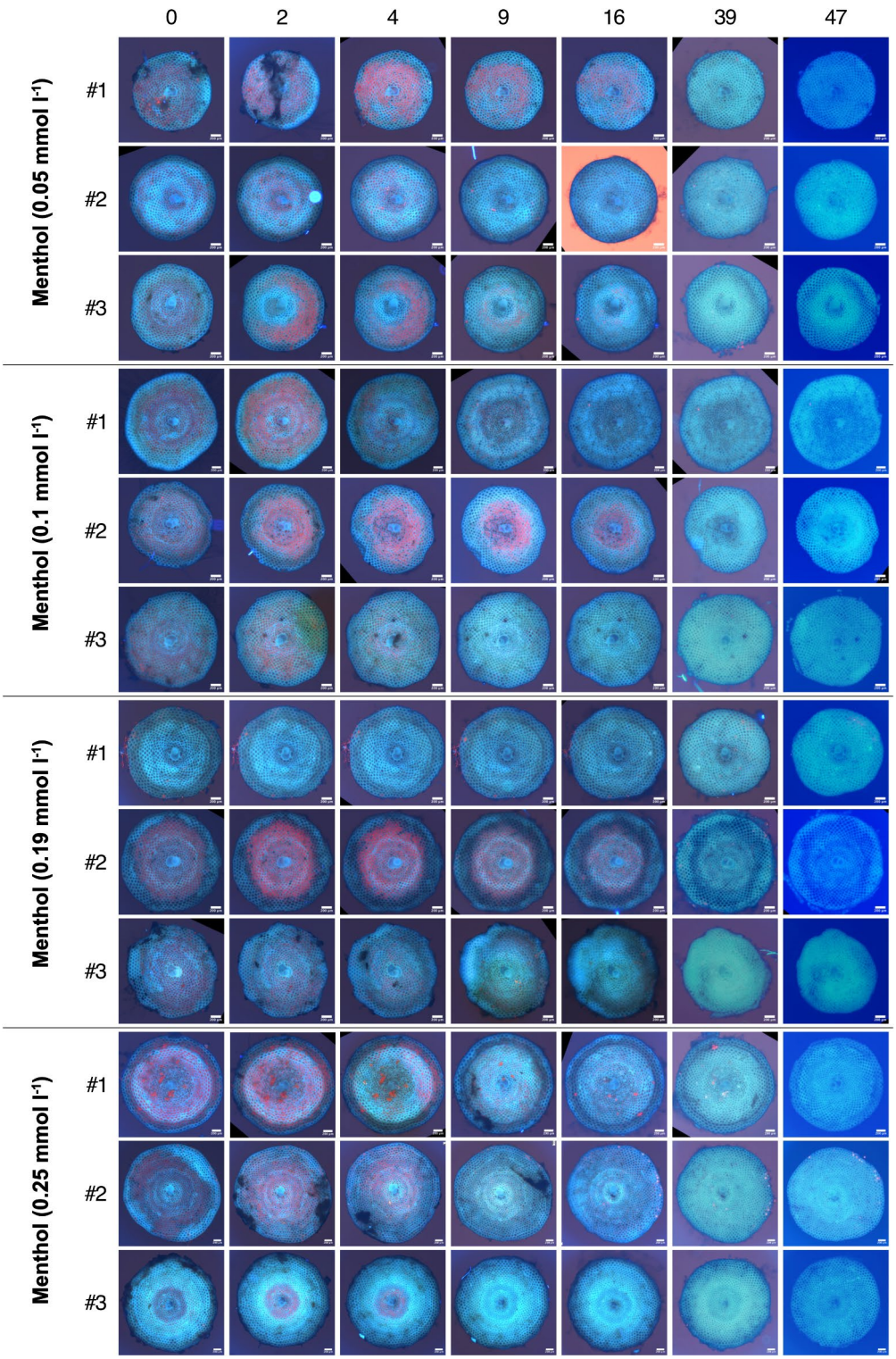

Fig. 2 Epifluorescence microscopy images of all *Sorites orbiculus* individuals during the 6-week MCC (Menthol Concentration Comparison) experiment at seven different time points (days) and for different menthol concentrations (mmol l-1) superskript -1. Scale bars are 200 µm.

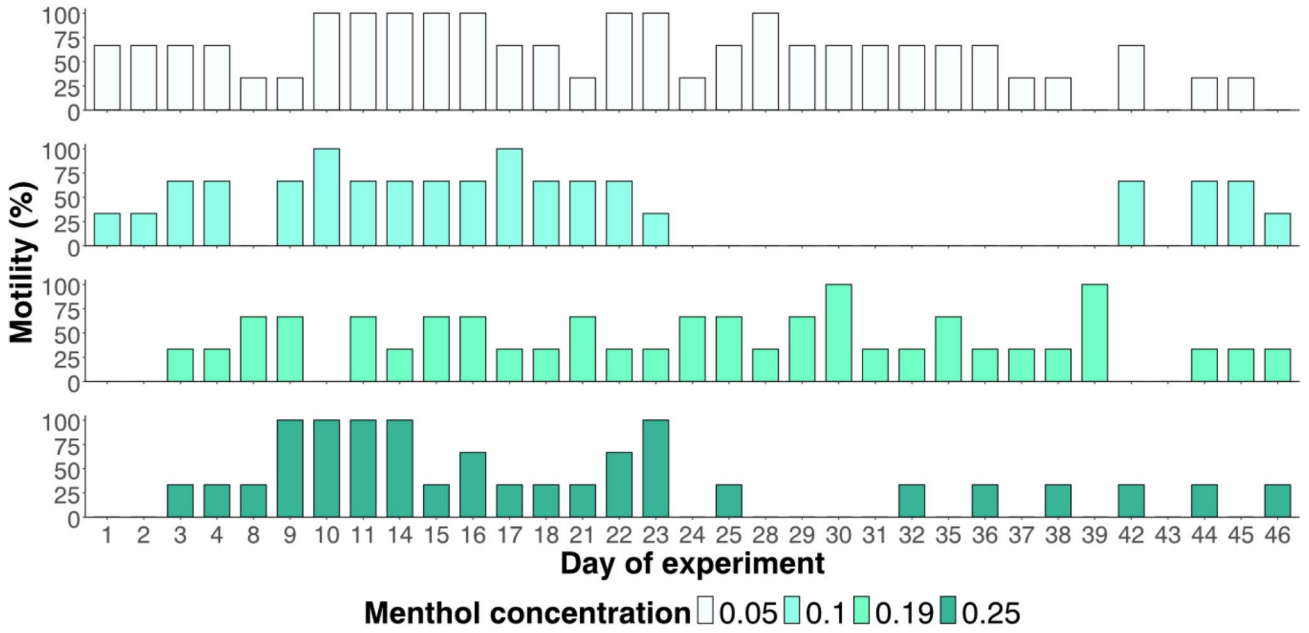

Fig. 3 Motility of *Amphistegina lobifera* during the 6-week MCC (Menthol Concentration Comparison) experiment, calculated as the number of individuals on the wall of the jar divided by the total number in the jar; $n=3$ *per* concentration. Menthol concentration given in mmol l^{-1}.

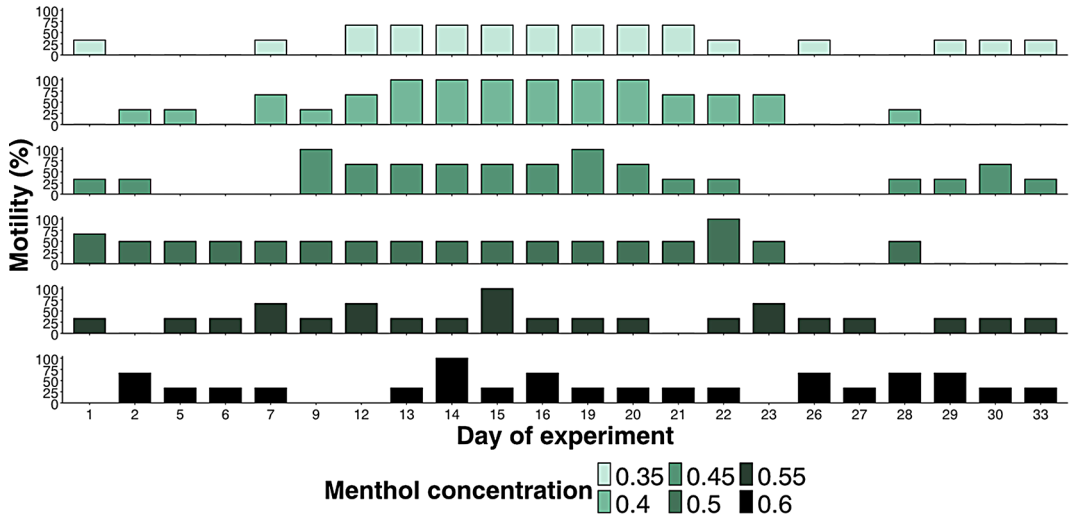

Fig. 4 Motility of *Amphistegina lobifera* during the HCT (High Concentration Trial) over 33 days. Motility is calculated as the number of individuals on the wall of the jar divided by the total number in the jar; $n=3$ *per* concentration. Menthol concentration given in mmol l^{-1}.

trial period, motility was quite low at <25–50%, and the foraminifera appeared more stressed than at the 0.19 mmol l^{-1} dose, which was then considered a safe dose to run a larger experiment with minimal mortality and still get effective results.

3.2 4-week bleaching ecophysiology assessment (MEA) measuring growth and mortality

Repeated menthol treatment at a concentration of 0.19 mmol l^{-1} induced symbiont loss in the MEA experiment; many specimens had no visible symbionts at the end of the experiment using confocal microscopy (Fig. 5a). For *A. lobifera*, all individually microscoped specimens lost 100% of their symbionts by the end of the experiment ($n=3$). For *S. orbiculus*, two out of three specimens (~66%) became completely aposymbiotic. The remaining specimens (~33%) still contained autofluorescent symbiont cells at the end of the experiment, as had been observed in some cases in the MCC experiment. Therefore, we suggest that individual selection and confocal microscopy of presumed aposymbiotic specimens is necessary prior to subsequent re-inoculation experiments in order to work with 100% aposymbiotic individuals.

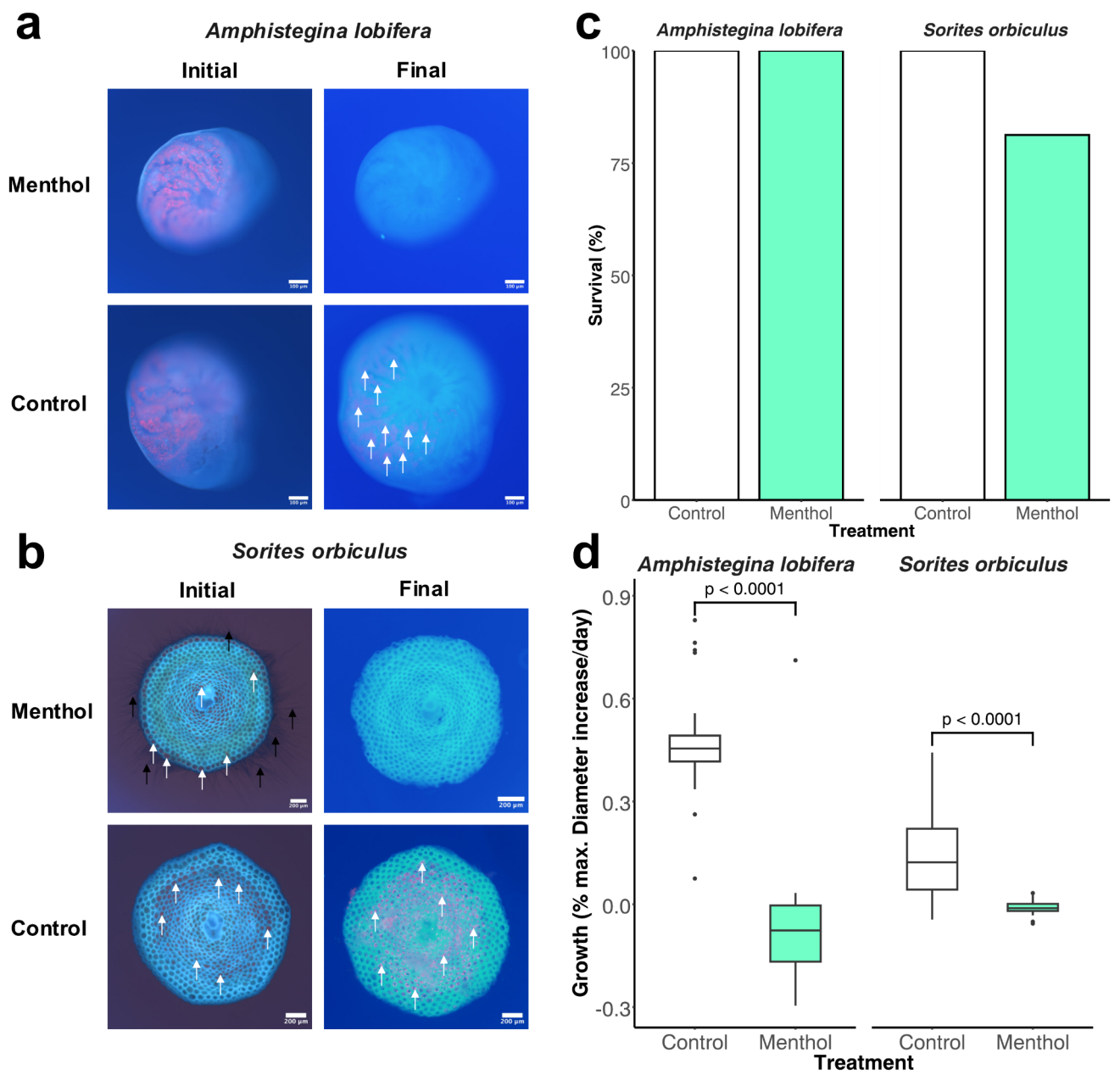

Fig. 5 Effects of menthol-DCMU exposure after the MEA (Menthol-bleaching Ecophysiology Assessment) 4-week experiment on the foraminiferan species *Amphistegina lobifera* and *Sorites orbiculus*. (**a**-**b**) Epifluorescence microscopy images of initial and final assessments showing symbiont loss (selected from $n = 3$ per treatment). Scale bars for *Amphistegina lobifera* and *Sorites orbiculus* are 100 µm and 200 µm, respectively. Fluorescing symbiont cells in the host cell are marked with white arrows, while pseudopodia of the foraminifera are indicated by black arrows. (**c**) Survival after the 4-week MEA experiment. (**d**) Outlier box plots of growth data given as % diameter increase *per* day. Significant differences between control and menthol treatment of the species is indicated by p-values (Mann-Whitney U test, for *A. lobifera*$n = 63$ for *S. orbiculus*$n = 32$).

Survival remained high in both species at the applied concentration (Fig. 5c). However, both species were stressed during the menthol-DCMU bleaching process as reflected in their growth rates.

Growth was significantly different between the control and menthol treatments in both species (Mann-Whitney U test, Table 1; Fig. 5d), indicating that menthol may reduce growth rates during the repeated cycle of applying menthol for bleaching in both species. While the growth of *A. lobifera* controls was in the normal range (Schmidt et al. 2016), no growth occurred in the menthol-DCMU bleaching treatment in either species, clearly indicating that they were under physiological stress. The negative growth rates of *A. lobifera* were likely caused by shell breakage, in

Table 1 Statistical results of the Mann-Whitney U test on growth rate comparison after the MEA (Menthol-bleaching ecophysiology Assessment)

Species	Compared groups	*n* (control)	*n* (menthol)	U	*p*-value
Amphistegina lobifera	Growth rate ~ Treatment	32	31	436	< 0.001
Sorites orbiculus	Growth rate ~ Treatment	16	16	102	< 0.001

the menthol-DCMU treatment. We speculate that the lack of symbiont-derived photosynthate limited calcification, which has been observed in reef-scale assessments linking coral bleaching to reduced coral growth (Bove et al. 2020; Davis et al. 2021).

For *S. orbiculus*, growth was low in the controls and in the menthol-DCMU treatment (Fig. 5d). Reduced symbiont activity may have negatively affected the growth of *S. orbiculus*. Our results confirm a study comparing lower and higher temperatures, which also induced reduced symbiont activity, and found 25 °C to be the optimal culture temperature for calcification (Kinoshita et al. 2021). For this experiment it was needed to maintain both species under the same light intensity, which is more detrimental to *S. orbiculus* than *A. lobifera*, as its dinoflagellate symbionts are more sensitive to a reduction in light levels than diatom symbionts. In future studies, growth of *Sorites orbiculus* could be stimulated at higher light levels between 50-100 PAR, which might increase symbiont activity in the controls but not stress on their photosystems.

Pseudopods, also known as reticulopods, are key structures in foraminifera that allow them to move, attach to surfaces, and capture food (Travis et al. 2002). Throughout the microscopy observations, pseudopods were occasionally visible in both the menthol and the control treatments. Clearly visible pseudopods were imaged (Fig. 6a-c) and videos of foraminifera in the aposymbiotic state with pseudopods are available as part of the dataset (deposited at https://www.pangaea.de/, https://doi.org/10.1594/PANGAEA.974618, Schmidt et al. 2025a). Due to the intermittent nature and variability of pseudopods in foraminifera (Greco et al. 2023), a better indicator of holobiont fitness is motility. This parameter could be assessed in *A. lobifera* as this species is generally very active compared to other benthic foraminifera, such as *S. orbiculus* (C.S. unpublished observations) and *P. calcariformata* (Schmidt et al. 2015).

Motility is a direct indicator of active pseudopodial movement and does not require detailed microscopic observation of each individual, and is known to decrease in response to thermal stress (Schmidt et al. 2011, Stuhr et al. 2018a, 2018b). Motility was not affected at a concentration of 0.19 mmol l^{-1} menthol (Fig. 6d), however after day 21, motility was higher in the control treatment than in the menthol treatment. This shows that menthol influenced motility. On the last day of the experiment (day 28), motility was significantly lower in the menthol treatment than in the control treatment (Table 2, Wald statistic, $p < 0.001$). There was also a significant interaction between menthol treatment and day of experiment (Wald statistic, Table 2, $p < 0.001$). This indicates that the predictor variable motility is not only influenced by the menthol treatment but also by the duration of the treatment. Due to the anaesthetic properties of menthol (Galeotti et al. 2001; Haeseler et al. 2002), repeated treatment with menthol-DCMU resulted in reduced motility. We suggest that menthol had a relaxing effect on pseudopodial activity. Tentacle relaxation and unresponsiveness due to menthol has been observed in several different marine species, such as pteropods (Yamazaki et al. 2024) and anemones (Matthews et al. 2016). Alternatively, reduced fitness in bleached specimens may result from the loss of symbiont-derived energy, as shown by proteomic evidence in thermally bleached Amphistegenids, where hosts compensated by utilizing alternative resources but still experienced decreased long-term fitness (Stuhr et al. 2018a, 2018b).

4 Conclusion

Our results show that large benthic foraminifera (LBF) harbouring diatoms and dinoflagellates can be experimentally induced to lose their symbionts (aposymbiosis) using a combination of menthol-DCMU as a bleaching agent. This process can be completed in about a month without causing lethal stress to the calcifiers. However, we observed negative effects on their fitness, as evidenced by lack of growth and reduced motility compared to the controls. Reduced fitness could be due to the treatment or due to the lack of the symbionts itself. The next experimental step is now to test whether menthol-treated LBF can be re-inoculated with different symbionts, and if their physiological health (e.g. normal growth, recovery of motility & calcification) can be restored to baseline levels. The experimental use of aposymbiotic foraminifera can provide valuable insights with broad implications for coral reef ecology, particularly in understanding adaptive strategies such as symbiont switching and the potential acquisition of novel, more thermally tolerant symbionts that could help these organisms cope with climate change. These experiments could include bleaching and re-inoculation with different microalgae, which could ultimately make the host more thermally tolerant, if the symbiont is the bottleneck to the thermal tolerance of the holobiont.

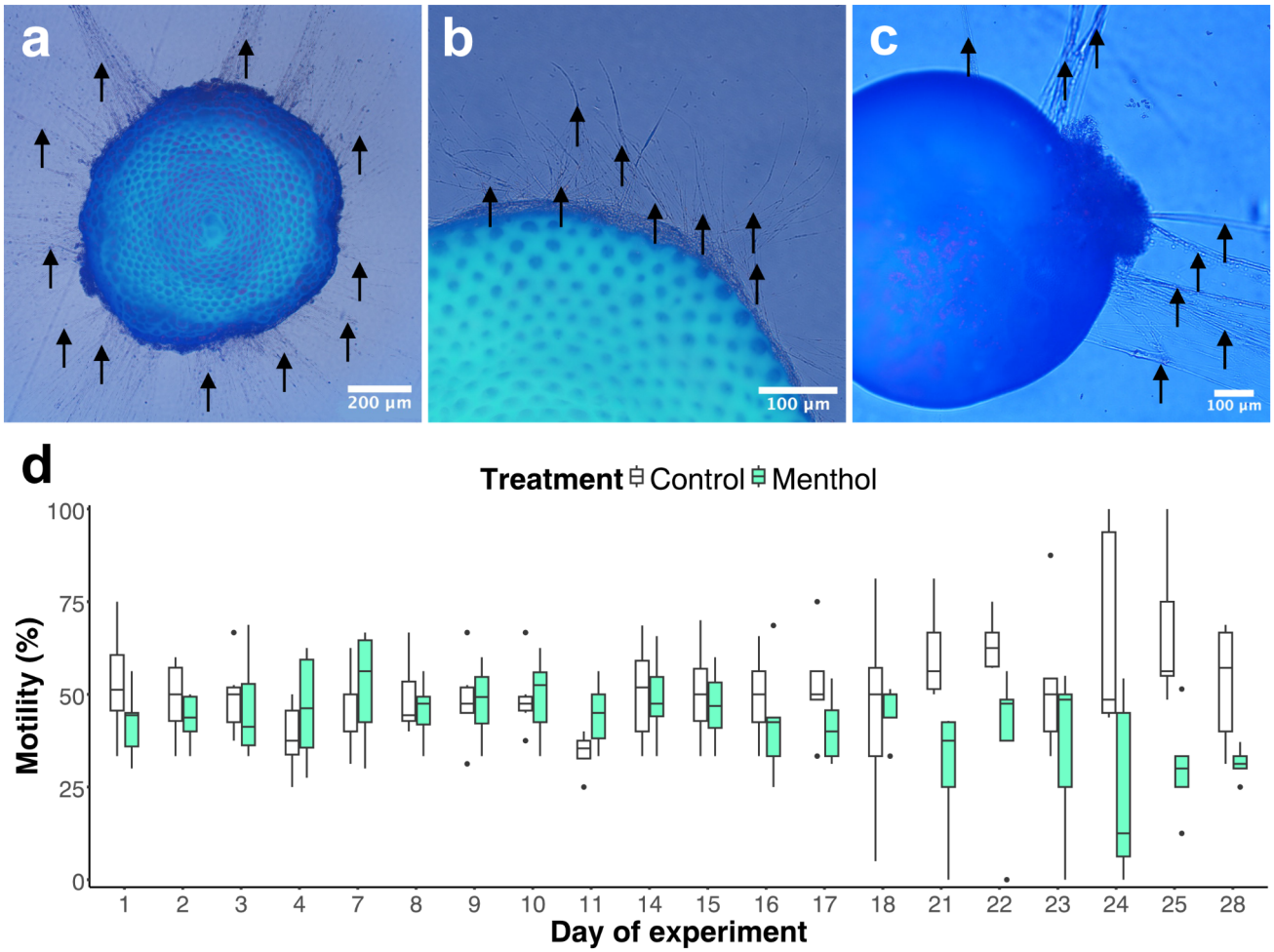

Fig. 6 Pseudopodial net of selected menthol-bleached and control specimens of *Sorites orbiculus* and *Amphistegina lobifera*, and motility of *A. lobifera* during the 4-week MEA (Menthol-bleaching Ecophysiology Assessment) experiment. (**a**) Extended pseudopodial net of *S. orbiculus* in the control treatment. (**b**) after the menthol-DCMU treatment (**c**) *A. lobifera* from the control treatment. Black arrows indicate pseudopodia. (**d**) Outlier boxplot showing changes of motility in *A. lobifera* over time ($n = 155$ *per* time point, grey boxes = controls and turquoise boxes = menthol treatment).

Table 2 Statistical results of the GLM (General linear Model) for the motility data of the 4-week MEA (Menthol-bleaching ecophysiology assessment) experiment including the Wald statistic.

Predictor variable	df	Wald chi-square	*p*-value
Day of experiment	19	15	0.73
Treatment	1	19.7	< 0.001
Treatment vs. Day of experiment	19	58.7	< 0.001

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Author contributions M.S. and D.S.R provided samples, C.S., X.P., C.A.O., and S.K.D designed and conceived the experiments. C.S., M.N. and D.N.P. R carried out the experiments and subsequent data analysis. C.S., M.S., D.S.R., X.P., C.A.O., D.N.P.R. and S.K.D. wrote and edited the manuscript.

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Data availability Datasets are uploaded to the PANGAEA (deposited at https://www.pangaea.de/, https://doi.org/10.1594/PANGAEA.974618, Schmidt et al. 2025b).

Declarations

Summary statement Novel application of a menthol-based method to generate symbiont-free benthic foraminifera, providing a valuable tool for studying symbiosis establishment and function in these ecologically important organisms.

Competing interests The authors declare no competing or financial

interests.

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