Symbiosis
https://doi.org/10.1007/s13199-026-01151-9

BRIEF REPORT

# Early experimental comparison of natural and assisted symbiont acquisition in *Stylophora pistillata*

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Received: 31 December 2025 / Accepted: 23 April 2026
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## Abstract

Acquisition of Symbiodiniaceae following a bleaching event is essential for coral recovery, yet most experimental approaches rely on cultured symbionts, which require specialized infrastructure and may exhibit laboratory-induced physiological alterations. Natural acquisition (NA), in which bleached corals acquire symbionts directly from healthy donors, represents a potentially relevant but understudied alternative. Here, we experimentally compared short-term recolonization dynamics under assisted acquisition (AA; laboratory-cultured symbiont cells) and natural acquisition (NA; passive transfer of released symbiont cells between coral nubbins) in menthol-bleached *Stylophora pistillata* over a three-week period. Partial symbiont recolonization was observed across all treatments, restoring approximately 10–15% of symbiont densities relative to unbleached controls. However, recolonization also occurred in the negative control, indicating that recovery was at least partly driven by residual symbionts remaining after bleaching. Accordingly, neither AA nor NA resulted in a statistically significant increase in symbiont density relative to the negative control. These findings suggest that, under the conditions tested, external symbiont supply did not clearly enhance early recolonization beyond background recovery. Instead, early recovery dynamics may be strongly influenced by residual symbiont proliferation. While NA remains a logistically simple and ecologically relevant approach, further optimization including increased replication, extended exposure time, and improved experimental conditions will be necessary to evaluate its potential in coral reinoculation studies.

**Keywords** Coral symbiosis · Symbiodiniaceae acquisition · Bleaching recovery · Assisted acquisition · Natural acquisition · *Stylophora pistillata*

## 1 Introduction

In recent decades, the accelerating frequency and intensity of marine heatwaves have triggered widespread coral bleaching, threatening the persistence of coral reef ecosystems. Bleaching constitutes the breakdown of a co-evolved symbiosis between reef-building corals and their photosynthetic Symbiodiniaceae, a partnership fundamental to coral metabolism and reef accretion (van Woesik et al. 2022). Consequently, the reestablishment of the coral–Symbiodiniaceae symbiosis following stress exposure is pivotal for coral survival and recovery and for shaping the future trajectories of coral reef ecosystems (Scharfenstein et al. 2022).

Over the past two decades, substantial research efforts have focused on experimentally promoting the reestablishment of coral–Symbiodiniaceae symbiosis following

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Published online: 30 April 2026

bleaching, using a range of artificial and laboratory-based approaches. Most experimental studies have relied on the direct administration of cultured algal strains, typically applied at concentrations of ~ $10^4$–$10^5$ cells/$mL^{-1}$ (Baumgarten et al. 2018; Scharfenstein et al. 2022). This approach, here referred to as Assisted Acquisition (AA), enables controlled assessment of symbiont uptake under standardized conditions. However, conventional culture-based approaches often require specialized infrastructure and long-term maintenance of Symbiodiniaceae under artificial conditions, which may alter algal physiology and affect host–symbiont interactions (Matthews et al. 2020; Suggett et al. 2017; Xiang et al. 2013). In addition, these microalgae exhibit broad physiological diversity and variable growth rates, making it challenging to obtain sufficient cell densities. Laboratory cultures may also contain associated microorganisms, potentially influencing experimental outcomes (Suggett et al. 2017).

Although adult corals are known to naturally acquire Symbiodiniaceae from surrounding seawater or nearby donor colonies (Coffroth et al. 2006, 2023; Quigley et al. 2017), this process, here defined as Natural Acquisition (NA), has received comparatively little attention in laboratory studies. Healthy coral colonies can act as natural reservoirs of Symbiodiniaceae, and the transfer of symbiont cells from donor colonies may provide an alternative to laboratory culture. In addition, NA may represent a more ecologically realistic pathway, as it involves symbionts released under natural conditions and potentially influenced by host-associated microbial communities (van Oppen and Blackall 2019; Zhang et al. 2021). However, the extent to which NA enhances symbiont recolonization relative to background recovery remains unclear.

Although previous studies have shown that bleached colonies can acquire Symbiodiniaceae from the environment or from donor sources via horizontal transmission (Coffroth et al. 2010; Nitschke et al. 2016; Scharfenstein et al. 2022), direct experimental comparisons between AA and NA under controlled conditions remain limited, particularly in adult corals. To address this gap, we compared early recolonization dynamics in menthol-bleached *Stylophora pistillata* nubbins exposed to laboratory-cultured symbionts (AA) or to healthy donor nubbins (NA) over a three-week period. Rather than assuming differential efficiency between approaches, this study aims to characterize short-term recolonization patterns and evaluate the extent to which external symbiont supply contributes to recovery under controlled conditions.

## 2 Materials and methods

### 2.1 Experimental setup

A total of 24 coral nubbins (~ 7 cm in length) were obtained by fragmenting six adult *Stylophora pistillata* colonies (four nubbins per colony) housed at the Genoa Aquarium (Italy), using shears. These colonies predominantly host microalgal endosymbionts (Symbiodiniaceae) as previously reported (Louis et al. 2025). Each nubbin was affixed to a 12-mm-diameter screw anchor using a two-part epoxy adhesive resin (Veneziani, Italy; Isa et al. 2024) and transferred to an indoor tank for a 4-week recovery period. The tank (3 × 1 × 0.7 m; 3100 L) was supplied with seawater via a continuous-flow pump system (Astralpool, Victoria Plus) operating at 8 $m^3$ $h^{-1}$, ensuring a complete water turnover every ~ 30 min. Before recirculation into the tank, seawater passed through a sand filter (Astralpool Artic; particle retention 0.4–2 mm) and a UV sterilizer (Panaque 750 S, equipped with four 40 W lamps). To maintain nubbin nutrition during the recovery period, a daily solution of 2 L containing the microalga *Tetraselmis* ($1.5 \times 10^6$ cells $mL^{-1}$) and rotifers (Phylum *Rotifera*; 250 individuals $mL^{-1}$, ~ 0.5 mm in size) was added to the tank. Environmental parameters were maintained at 26 °C, 36 PSU salinity, and ~ 200 µmol photons $m^{-2}$ $s^{-1}$ under a 12 h:12 h light–dark cycle.

Following the recovery period and prior to the start of the experiment, six nubbins (one from each adult mother colony) were used for symbiont isolation (see below). To establish baseline symbiont densities, three nubbins were randomly sampled for symbiont quantification (Control treatment, C). Twelve nubbins were then randomly selected and subjected to menthol-induced bleaching (see below). After a 5-day recovery period in additional tanks containing menthol-free water, three of these bleached nubbins were sampled to determine symbiont densities (Bleached treatment, B). The remaining nine bleached nubbins were allocated to the experimental treatments. Specifically, three nubbins were assigned to each of the following groups: (*i*) assisted acquisition (AA), (*ii*) natural acquisition (NA; co-maintained with six unstressed nubbins in a 3:1 volume ratio, as reported below), and (*iii*) a negative control (NC; bleached corals without symbiont administration). All experimental nubbins were maintained for three weeks in 10-L Ferplast containers (35 × 20 × 23 cm) filled with sand-filtered, UV-sterilized, and boiled (40 min) hereafter referred to as sterile seawater. Finally, three unstressed nubbins were maintained under identical conditions and served as a positive control (PC), allowing us to account for any effects associated with maintenance in 10-L containers independent of bleaching or inoculation procedures.

During the experimental period, approximately 30% of the water volume in each tank was replaced every two days with freshly prepared, sterile seawater to maintain water quality and prevent the accumulation of metabolic products. Following each water change, symbiont cells were re-administered in the AA treatment by adding sterile seawater containing approximately $10^5$ cells $mL^{-1}$. Water quality parameters, including salinity, pH, ammonium, and nitrite, were regularly monitored throughout the experiment to ensure stable conditions across treatments. Water chemistry remained stable throughout the experiment. Specifically, salinity ranged from 35.5 to 36.7 PSU, while pH remained between 7.9 and 8.0. Moreover, ammonia and nitrite concentrations were consistently below 0.01 mg $L^{-1}$. Nubbins were not fed during the experimental period.

### 2.2 Symbiont cell isolation

To isolate Symbiodiniaceae cells, a ~ 3 cm fragment was collected from each of the six aforementioned adult *S. pistillata* colonies ($n = 6$) using shears. The fragments were pooled and incubated for 2 h at room temperature in a 50-mL Falcon tube containing 30 mL of calcium- and magnesium-free artificial seawater (Li et al. 2020; Roger et al. 2021) to induce symbiont cell release. Cell isolation was confirmed by optical microscopy through visualization of released Symbiodiniaceae cells, after which the cells were cultured in 5-L glass cylinders containing sterile seawater (sand-filtered, UV-sterilized, and boiled for 40 min) supplemented with f/2 medium (Guillard and Ryther 1962). Cultures were maintained at 23 °C under a 12 h:12 h light–dark cycle (light from 6:00 to 18:00; dark from 18:00 to 6:00) at 80 µmol photons $m^{-2}$ $s^{-1}$ (Baumgarten et al. 2013). Salinity was initially adjusted to 30 PSU and then gradually increased through evaporation to 36 PSU, matching the conditions at the Genoa Aquarium. Prior to downstream applications, cultures were inspected microscopically to verify the absence of visible bacterial, protistan, or other microalgal contaminants.

### 2.3 Menthol bleaching procedure and morphological evaluation of coral nubbins

To artificially induce bleaching in the twelve randomly selected nubbins, a menthol-induced bleaching procedure was applied following established protocols for cnidarians, including *S. pistillata* (Wang et al. 2012; Matthews et al. 2016; Scharfenstein et al. 2022). Briefly, menthol (Sigma-Aldrich, St. Louis, MO, USA) was added to sterile seawater to achieve a final concentration of 0.38 mM (Wang et al. 2012). Nubbins were incubated for 8 h (approximately 08:30 to 16:30), corresponding to most of the daytime photoperiod (Scharfenstein et al. 2022), in a 10-L Ferplast container filled with menthol-enriched sterile seawater. This was followed by a 16 h incubation in fresh, menthol-free sterile seawater in a second 10-L Ferplast container. Following the bleaching treatment, nubbins were transferred to menthol-free 10-L Ferplast containers filled with sterile seawater and monitored daily over a 5-day recovery period. Recovery was evaluated based on the reappearance of mesenterial filament extrusion and active polyp extension, which were considered indicators of physiological readiness for the symbiont inoculation step. Bleaching progression and nubbin condition were assessed once per day, beginning after the first menthol incubation (~ 17:00) and continuing at 24-h intervals during the recovery and experimental phases, following the criteria described by Isa et al. (2023). In particular, the following traits were recorded: (*i*) mortality; (*ii*) visual health status using the Coral Health Chart (Siebeck et al. 2006); (*iii*) extent of mucus production; (*iv*) proportion of retracted polyps and (*v*) proportion of tissue exhibiting necrosis. Each trait was scored independently by two operators on a scale from 0 (no visible stress) to 3 (severe stress).

### 2.4 Assisted Acquisition (AA)

In the AA treatment, previously isolated and cultured Symbiodiniaceae cells were administered to bleached nubbins by adding a suspension adjusted to ~ $10^5$ cells $mL^{-1}$ (Baumgarten et al. 2018; Scharfenstein et al. 2022; Bouwmeester et al. 2022).

### 2.5 Natural Acquisition (NA)

In the NA treatment, bleached nubbins were placed next to six healthy nubbins at a donor-recipient ratio of 1:3 (based on unpublished preliminary observations). Because *S. pistillata* nubbins have a roughly cylindrical morphology, the surface area of each nubbin was calculated by separating the main body of each nubbin and, when present, the branches. The total surface area was then obtained by summing the surface area of each component according to the formula:

$$S\,total = \sum_{i=1}^{n} (\pi\, d_i h_i)$$

where S is the total surface, $d_i$ is the diameter of nubbins main body and branches, $h_i$ is the nubbin/branches height (first measurement). Nubbins were co-maintained under these conditions for the entire experimental period (three weeks), allowing continuous exposure to naturally released symbiont cells.

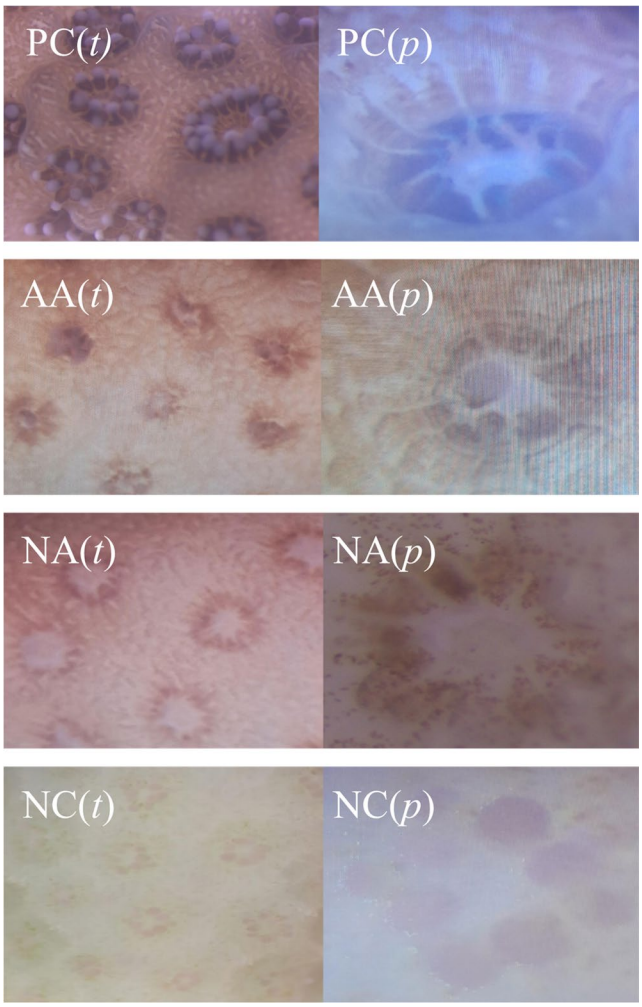


**Fig. 1** Microscopy images showing qualitative differences in *Symbiodiniaceae* abundance across treatments. Panels show representative microscope images of coral tissue (*t*) and polyp regions (*p*) corresponding to the positive control (PC), assisted administration (AA), natural administration (NA), and negative control (NC) treatments, respectively. Darker pigmentation reflects higher *Symbiodiniaceae* abundance, whereas pale or translucent areas indicate reduced or absent symbiont presence

### 2.6 Symbiodiniaceae cell density count

Symbiont density was quantified following the general approach described by Louis et al. (2025). Briefly, coral tissue was removed from each nubbin by airbrushing using filtered compressed air, and the resulting tissue slurry was homogenized by gentle vortexing. *Symbiodiniaceae* cells were counted using an Improved Neubauer hemocytometer under an optical microscope at 400× magnification. For each sample, five independent hemocytometer sub-counts were performed to improve counting accuracy, as recommended by Louis et al. (2025). Cell density (cells $mL^{-1}$) was then converted to cells $cm^{-2}$ by normalizing to the total surface area of each nubbin. All statistical analyses were performed in R (version 4.2.2). Because *Symbiodiniaceae* densities exhibited right-skewed distributions, data were $log_{10}$-transformed prior to testing to meet normality and homoscedasticity assumptions. A one-way ANOVA was used to test for differences in symbiont density across treatments (C, B, PC, NC, NA and AA). Model residuals were inspected to verify ANOVA assumptions. Tukey's HSD post hoc test was used to assess pairwise differences. Transformed data were visualized using a $log_{10}$-scaled y-axis boxplot showing median, quartiles, and individual biological replicates.

**Table 1** Symbiont cell abundance across experimental treatments. The table reports average symbiont cell counts (cells per hemocytometer field), total symbiont cells extracted from each coral fragment, fragment surface area ($cm^2$), and symbiont cell densities normalized per unit area (cells $cm^{-2}$). The final column expresses normalized symbiont densities in units of $\times 10^6$ cells $cm^{-2}$. Samples correspond to the experimental treatments described in the study (C = control; B = bleached; PC = positive control; NC = negative control; NA and AA = natural and assisted symbiont administration, respectively)

| Sample_IDs | Average cell count | Total cells on coral fragments | Surface area (cm2) | Cells/cm2 | Cells/cm2 x 10^6 |
|---|---|---|---|---|---|
| C1 | 73.2 | 4,392,000 | 2.16 | 2,035,565 | 2.036 |
| C2 | 89.1 | 5,346,000 | 3.67 | 1,457,629 | 1.458 |
| C3 | 85.65 | 5,139,000 | 2.81 | 1,826,696 | 1.827 |
| B1 | 4.1 | 246,000 | 2.47 | 99,572 | 0.100 |
| B2 | 2.05 | 123,000 | 3.39 | 36,289 | 0.036 |
| B3 | 2.6 | 156,000 | 2.85 | 54,774 | 0.055 |
| CP1 | 72.75 | 4,365,000 | 1.94 | 2,245,312 | 2.245 |
| CP2 | 101.9 | 6,114,000 | 3.71 | 1,649,163 | 1.649 |
| CP3 | 78.35 | 4,701,000 | 3.44 | 1,364,949 | 1.365 |
| CN1 | 4.95 | 297,000 | 2.88 | 103,202 | 0.103 |
| CN2 | 7.85 | 471,000 | 2.58 | 182,570 | 0.183 |
| CN3 | 7.55 | 453,000 | 2.40 | 188,670 | 0.189 |
| NA1 | 10.25 | 615,000 | 3.31 | 186,081 | 0.186 |
| NA2 | 3.65 | 219,000 | 3.03 | 72,352 | 0.072 |
| NA3 | 35.05 | 2,103,000 | 4.07 | 516,717 | 0.517 |
| AA1 | 15.65 | 939,000 | 4.36 | 215,465 | 0.215 |
| AA2 | 22.15 | 1,329,000 | 4.99 | 266,395 | 0.266 |
| AA3 | 17.85 | 1,071,000 | 2.78 | 385,458 | 0.385 |

## 3 Results

### 3.1 Coral bleaching assessment

Menthol-induced bleaching caused a drastic reduction in symbiont density (Fig. 1; Table 1). Control nubbins (C) displayed an average *Symbiodiniaceae* density of approximately $1.8 \times 10^6$ cells $cm^{-2}$, whereas bleached nubbins (B) showed a mean reduction to $\sim 6.3 \times 10^4$ cells $cm^{-2}$ (Table 1), corresponding to only ~3–4% of control symbiont densities. Morphological assessments during the 5-day recovery period indicated that some effects of menthol exposure

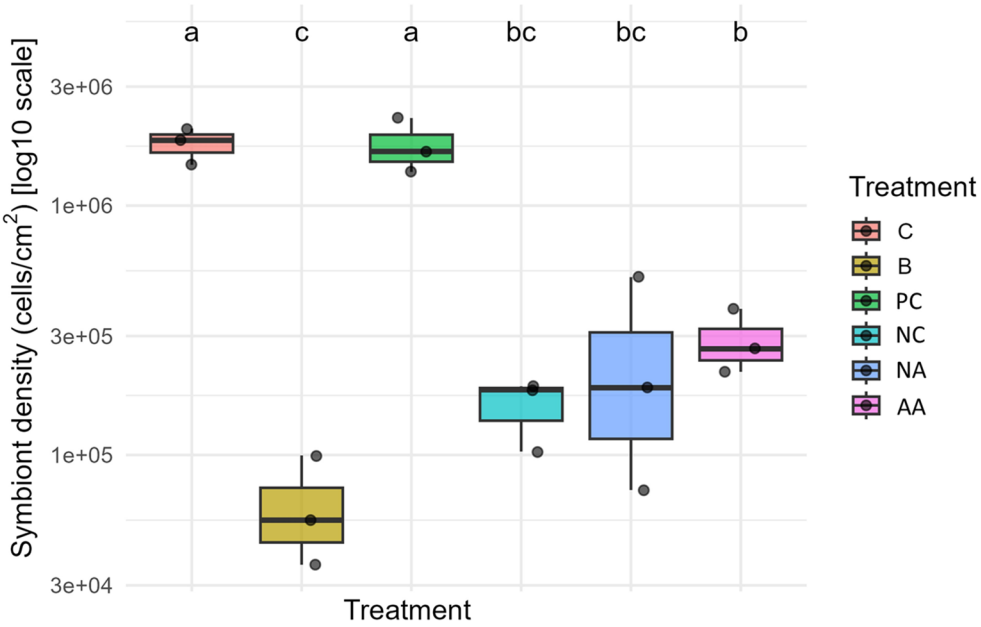


**Fig. 2** Symbiont cell densities across treatments after three weeks of recovery. Boxplot showing the distribution of *Symbiodiniaceae* cell densities (cells $cm^{-2}$) in coral nubbins following bleaching and subsequent treatments. Control groups (C and PC) maintained high symbiont densities, while bleached nubbins (B) and negative controls (NC) remained depleted. Inoculation treatments (NA and AA) resulted in partial recolonization, with AA showing the highest recovery among the bleached groups. Letters indicate statistical groupings based on one-way ANOVA followed by Tukey's post-hoc test ($p < 0.05$)

persisted temporarily after treatment cessation. However, qualitative indicators including re-establishment of polyp extension and reduced mucus production were observed by the end of this period, suggesting that nubbins had recovered to a physiological condition suitable for subsequent *Symbiodiniaceae* administration.

### 3.2 Symbiont recolonization comparison between treatments

Symbiont densities differed significantly among treatments (one-way ANOVA, $F(5,12) = 34.0$, $p < 0.001$). Tukey's HSD indicated that both control groups (C and PC) maintained significantly higher symbiont densities than all bleached treatments, confirming that maintenance in 10-L containers did not affect symbiont stability over three weeks.

Negative control nubbins (NC) exhibited partial recovery relative to bleached nubbins (B), although this increase was not statistically significant. Symbiont densities in NC reached approximately ~ 9% of control values, indicating regrowth of residual symbionts (Fig. 2; Table 1).

Among the recolonization treatments, AA showed a significant increase in symbiont density relative to B. NA exhibited a comparable but non-significant increase relative to B and did not differ statistically from NC, reflecting higher within-group variability (Fig. 2). Notably, neither AA nor NA resulted in a statistically significant increase in symbiont density relative to the negative control. Quantitatively, both treatments restored symbiont densities to approximately $1.0–3.0 \times 10^5$ cells $cm^{-2}$, corresponding to ~ 10–15% of values observed in healthy controls (Table 1).

## 4 Discussion

This study provides an early assessment of symbiont recolonization dynamics in bleached *Stylophora pistillata*, comparing natural (NA) and assisted (AA) acquisition over a short, 3-week experimental period. Given that symbiont acquisition in *S. pistillata* typically occurs over timescales of several weeks to months (Nakamura et al. 2003; Scharfenstein et al. 2022; Sikorskaya et al. 2025), the results presented here should be interpreted as early-stage responses rather than equilibrium conditions.

Symbiont recolonization was observed across all treatments, including the negative control, indicating that recovery was at least partly driven by residual symbionts remaining after bleaching. Although both AA and NA resulted in partial increases in symbiont density (~ 10–15% of control values), neither treatment produced a statistically significant increase relative to the negative control. These findings indicate that, under the conditions tested, external symbiont supply did not clearly enhance early recolonization beyond background recovery. Instead, early recovery dynamics appear to be mainly influenced by the proliferation of residual symbionts, likely associated with their more favorable *in hospite* physiological condition.

Differences between acquisition pathways were nevertheless observed in the variability of responses. NA exhibited greater variability among replicates, suggesting that natural acquisition may be influenced by colony-specific traits or microenvironmental conditions affecting symbiont release and uptake. In contrast, AA resulted in a more consistent response, likely reflecting the controlled delivery of symbiont cells. However, this consistency did not translate

into a clear advantage in terms of overall symbiont density relative to background recovery.

Despite the absence of a measurable enhancement in recolonization relative to the negative control, NA remains of interest from a methodological perspective. Because NA relies on symbionts naturally released by healthy donor colonies, these cells may retain physiological characteristics closer to the *in hospite* state. In addition, NA requires minimal manipulation compared to culture-based approaches, potentially reducing logistical constraints associated with symbiont isolation, maintenance, and delivery. However, the extent to which these factors influence recolonization success remains unclear and warrants further investigation.

Conversely, AA involves the use of cultured symbionts, which may exhibit physiological differences compared to their *in hospite* counterparts. Previous studies have shown that cultured Symbiodiniaceae can undergo shifts in metabolic state, lipid composition, and stress responses during in vitro growth (Klueter et al. 2015; Chen et al. 2015; Pasaribu et al. 2015). Such changes may affect their capacity to establish symbiosis, particularly during early recolonization stages. In the present study, AA was performed using recently isolated and subsequently cultured symbionts rather than long-term maintained strains, which may partially mitigate these effects. Nevertheless, the role of symbiont physiological state in determining recolonization success requires further investigation.

Our results also highlight the importance of host condition in shaping recolonization dynamics. Menthol bleaching effectively reduced symbiont densities by approximately 96–97%, in line with previous studies (Wang et al. 2012; Matthews et al. 2016; Scharfenstein et al. 2022), but did not result in complete symbiont removal. Residual symbionts likely contributed to the recovery observed across all treatments. In addition, menthol exposure induced transient physiological stress, including reduced polyp activity, tissue contraction, and increased mucus production, which persisted for several days after treatment. These factors may have influenced the capacity of corals to acquire or retain new symbionts during the early recovery phase.

Preliminary observations conducted prior to the experiment indicated that bleached nubbins can be particularly vulnerable under laboratory conditions, occasionally exhibiting mortality and signs of microbial overgrowth. Although these observations were not quantified, they underscore the importance of maintaining strict environmental control during bleaching and recovery. In this context, the use of sterile seawater may help reduce the risk of colonization by opportunistic microorganisms, which are known to proliferate in stressed corals and interfere with recovery processes (Boilard et al. 2020; Savary et al. 2021).

Finally, we observed that maintaining corals in small (10-L) experimental containers for extended periods led to signs of stress, despite stable water quality parameters. Although tank volume is rarely considered a primary experimental variable, limited water volume may reduce environmental buffering capacity and increase the sensitivity of corals to subtle fluctuations. Methodological guidelines generally recommend the use of larger tanks for long-term experiments to ensure greater environmental stability (Bartlett 2013), suggesting that tank size should be carefully considered in future experimental design.

Overall, our findings emphasize the importance of residual symbionts in driving early recovery dynamics and highlight the need for caution when interpreting short-term inoculation experiments. While NA represents a logistically simple and ecologically relevant approach, further optimization, including increased replication, extended exposure periods, and improved experimental conditions, will be necessary to evaluate its potential role in coral recovery. Future studies incorporating longer timescales and more complex environmental conditions will be essential to determine whether natural acquisition can contribute meaningfully to post-bleaching recovery in reef ecosystems.

**Author contributions** Experimental work was conducted by VI, AM, EM, LMT, GDR, AC and FC. Provision of infrastructure, aquarium facilities, and coral biological material was ensured by PG, DS, YDL and SL. The first draft of the manuscript was written by VI, AM, and FC. Funding acquisition was secured by FC, MM, and MEM. All authors contributed to the revision process, provided critical feedback, and approved the final version of the manuscript.

**Funding** Open access funding provided by Università degli Studi di Padova within the CRUI-CARE Agreement. This research was funded by the Italian Ministry of University and Research (MUR) under the National Recovery and Resilience Plan (PNRR), Mission 4, Component 2, Investment 1.2, Project SOE2024_0000074, CUP C13C24001130006, funded by the European Union - NextGenerationEU.

**Data availability** All data generated or analyzed during this study are included in this published article. The raw datasets are available from the corresponding author on reasonable request.

## Declarations

**Ethics approval** Coral fragments were obtained and maintained in accordance with institutional and aquarium regulations. No field collection requiring governmental permits was conducted.

**Competing interests** The authors declare no competing interests.

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