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A pioneering longterm experiment on mesophotic macrofouling communities in the North Atlantic

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The mesophotic zone represents one of our planet's largest and least explored biomes. An increasing number of studies evidence the importance of macrofouling species in marine ecosystems, but information on these communities and the factors influencing their structures at mesophotic depths remain poor. This lack of understanding limits our ability to predict anthropogenic impacts or conduct restoration operations in the mesophotic and the lower boundary of the euphotic zones. In this study, we performed a 24-month experiment in a natural environment to investigate three factors influencing the macrobenthic community structure of the mesophotic and the euphotic lower boundary: depth, substrate orientation and substrate material. Using a manned submersible, several recruitment panels of two different materials were deployed at 100, 200 and 400 meters in vertical and horizontal positions. All three factors contributed to structuring the macrofouling communities, but depth and substrate orientation displayed the strongest effects. This study not only advances our understanding of lower boundary euphotic and mesophotic macrofouling communities but also establishes a foundation for future research and restoration efforts of mesophotic environments in the Madeira archipelago, where mesophotic habitats are amongst the least studied marine habitats in the Northeast Atlantic.

The ocean covers more than 70% of the Earth's surface[1], with most of this area covering depths over 100 m, thus representing our planet's most extensive biome. Despite the size, mesophotic and deep-sea habitats in general (>200 m depth) remain the least explored environments on Earth[2,3]. In the archipelagos of Macaronesia (NE Atlantic), mesophotic habitats still need to be explored, with most studies and efforts to date constrained to the Azores archipelago and the Canary Islands[4–8]. Located between these two archipelagos and 700 km from Africa, Madeira mesophotic habitats may be particularly interesting from biogeographical and ecological viewpoints. However, most of the current knowledge on biological diversity around the Madeira archipelago comes from experimental and scientific fishing trials[9,10] and a few oceanographic expeditions that used pelagic and bottom contact gears for sampling (e.g.[11–13]). Beyond this, only a limited number of studies

have conducted video and/or photo surveys to assess biodiversity and document local deep-sea habitats in this region[14–18].

During the most recent efforts, several mesophotic and deep-sea benthic communities around Madeira were recorded for the first time. These include communities highly vulnerable to anthropogenic activities, such as a mesophotic kelp forest and a coral garden populated by *Paramuricea* cf. *grayi*, discovered in the Madeira-Desertas Ridge[14]. In that study, it is argued that the steep environmental gradient found at the ridge [i.e. long-distance range (>100 m depth) of sunlight penetration, high topographic variation and heterogeneous substrates] largely contributed to the establishment of several biotopes with high biological diversity[14].

In an era where anthropogenic impacts continue to rise and compromise the health of the marine ecosystem and where interest in deep-sea

mining is growing alongside demand for minerals to support the green transition[19], it is of utmost importance to better understand their compounding effects and how to mitigate their impacts. While no commercial deep-sea mining has yet taken place on continental shelves, current plans to start deep-sea mining operations across the globe lead to a growing urgency to understand recruitment and succession in mesophotic and deep-sea habitats.

Sessile community succession generally begins with the growth of a biofilm of microbial communities, mainly composed of bacteria (microfouling) that facilitate the settlement of invertebrate larvae (macrofouling). These larvae then grow and gradually occupy the available space[20–22]. Similar to what happens in shallow waters, euphotic lower boundary and mesophotic macrofouling assemblages may include a wide range of sessile species, including bryozoans, hydrozoans, sponges, molluscs, polychaetes, crustaceans and tunicates[22]. Their community composition and organization are strongly correlated to light, temperature, pressure, food and larvae availability, and therefore greatly influenced by depth[3,23,24]. The employment of settlement plates has been widely used in shallow waters globally[25–27] to study the effect of multiple factors (anthropogenic or natural) on benthic communities. Being simple, low-cost, and easily replicable, settlement plates have become a standard research tool for monitoring and comparing fouling communities and succession across time and space[27].

However, compared with the numerous experimental settlement studies performed in shallow coastal areas[25,28–31], documentation on fouling communities below 50 m remains rare and geographically scarce[22,23,32]. This lack of information can greatly hamper conservation efforts, management strategies and future restoration efforts, considering the importance of understanding early recruitment and ecological succession in the assemblage of sessile communities.

Benthic organisms need favorable settlement conditions to colonize new habitats[33]. As such, the larvae of several species have been found to spend more time in the planktonic phase with increasing depth to maximize the possibility of finding a suitable substrate to colonize, swimming in the water column for months to over a year[34]. Furthermore, many pelagic larvae exhibit active settlement preferences (e.g.[35] and references therein), so factors not depth-related can also affect their adult community structure, including substrate orientation[36–38] and type of material[35–37]. These physical, ecological and temporal parameters may all play a role in shaping euphotic lower boundary and mesophotic benthic assemblages.

To date, only a few studies have used settlement plates in the mesophotic zone (e.g.[23,39–48]), and even fewer studies have considered the synergistic effect of multiple factors on mesophotic macrofouling community richness and structure (e.g.[40,48–51]).

In addition to variations in natural conditions such as depth and orientation, understanding how sessile mesophotic organisms colonize and grow on artificial and rocky substrates will be key when assessing risks posed by plastic pollution, infrastructure, mining operations and in designing restoration practices (e.g. artificial reefs, recruitment and transplantation).

In this context, the current study was designed to examine the macrofouling community settlement on experimental substrata after ~ 24 months of exposure along a depth gradient (100–400 m). The main objectives of this initial study were: i) to investigate the effect of depth (i.e. temperature, light, pressure) on the distribution of the macrofouling assemblages; ii) to assess how macrofouling assemblages respond to differences in substrate orientation and; iii) type of material (i.e. plastic based vs natural rock). We further hypothesize that the diversity and abundance of fouling communities will decrease with depth and be favored by artificial substrates and vertical orientation.

Using modular artificial units to assess differences in epibenthic macrofauna colonizing hard substrates, this pioneering experimental study in Madeira makes an important contribution to our present understanding and the future conservation and restoration of mesopelagic habitats in the North Atlantic.

Results

Taxonomic composition and species diversity of the epibenthic macrofauna community

A total of 49 taxa representing 11 different phyla colonized experimental plates: Annelida (15), Arthropoda (2), Brachiopoda (2), Bryozoa (13), Chordata (3), Ciliophora (1), Cnidaria (5), Entoprocta (1), Foraminifera (2), Mollusca (1), Porifera (4) (see details on Supplementary Table 1 and Supplementary Data 3). In particular, 40 taxa were found at 100 m, 23 at 200 m, and 18 at 400 m (for the detailed list, see Supplementary Table 1 and Supplementary Data 3).

The Annelida and Foraminifera were the dominant phyla at all depths, and the species most influenced by tested drivers were the foraminifera *Elphidium crispum* and the annelid *Salmacina incrustans* (Fig. 1). Among the identified taxa, there were six newly recorded species for Madeira, including *E. cripsum* (Supplementary Data 3). Additionally, a cross-inspection of the settlement plates' biodiversity revealed that five taxa were exclusively detected in natural substrate (basalt), whereas seven taxa were exclusively detected on plastic-based substrate (i.e. PVC) (Supplementary Data 3).

Factors shaping distinct macrofouling assemblages

At the end of the experiment, both the number of species and their mean coverage were highest at the shallower site (100 m) compared to the deeper sites (200–400 m) (Fig. 2A). Species richness ranged from 6 to 18 at 100 m (mean ± SD: 10.5 ± 3.1), 3 to 6 at 200 m (4.2 ± 1.5), and 1 to 10 at 400 m (5 ± 2.1). Mean coverage varied from 19.2 to 94.9% at 100 m (58.5 ± 25.8%), 2 to 28.3% at 200 m (13.6 ± 6.9%), and 1 to 42.4% at 400 m (16.1 ± 9%).

Substrate orientation also affected species richness and coverage, with the most pronounced differences observed at 100 m depth (Supplementary Table 2). Vertical plates at this depth had the highest species richness (vertical (V): 12.3 ± 3 vs. horizontal (H): 8.7 ± 1.9) and mean coverage (V: 81 ± 11% vs. H: 36.1 ± 13.2%). Conversely, at 400 m, horizontal plates exhibited higher species richness (H: 5.8 ± 1.9 vs. V: 4.5 ± 2) and mean coverage (H: 19.7 ± 9.3% vs. V: 12.5 ± 7.4%). However, this trend was more robust in shallower (100 m) than in deeper depths (200–400 m) (Fig. 2B).

A Distance-based Linear Mode (DistLM) confirmed that all three variables contributed to explaining the variation in the macrofouling assemblages (Table 1). DistLM results also show that depth had a greater influence in shaping the communities, followed by substrate orientation and, finally, type of material; this can also be illustrated by a Principal Coordinate Ordination (PCO) plot where these co-variables have been imposed as vectors and a metric Multidimensional Scaling (mMDS) (Figs. 3, 4). DistLM further identified that the interplay of depth, substrate orientation, and type of material provides the best combination of explanatory co-variables (Table 1).

PERMANOVA outputs indicated that all three factors (i.e. depth, substrate orientation and type of material) were significant in shaping fouling communities, resulting in assemblages that were significantly different when grouped by each of the factors alone or combined, with one exception. Fouling assemblages were not significantly different for substrate orientation versus type of material groupings (Supplementary Table 3).

The PCO plot also illustrated overall variations in the five taxa that most contributed to such ordination: *S. incrustans* (Annelida) and *E. crispum* (Foraminifera), followed by the Terebellidae (Annelida), the Hesionidae (Annelida) and *Callopora* sp. (Bryozoa). An inspection of the PCO plot suggests that *S. incrustans* and *E. crispus* were more influenced by the orientation of the plates, Hesionidae by the depth, Terebellidae by the interplay of these two variables (orientation and depth), and *Callopora* sp. appeared to be more influenced by the type of material (Fig. 3).

While PERMANOVA analysis identified a significant influence of all the considered factors/co-variables (Supplementary Table 3), pairwise analysis of similarity (ANOSIM) identified which assemblages were different and which ones had no significant differences when comparing fouling assemblage composition between all possible pairwise combinations
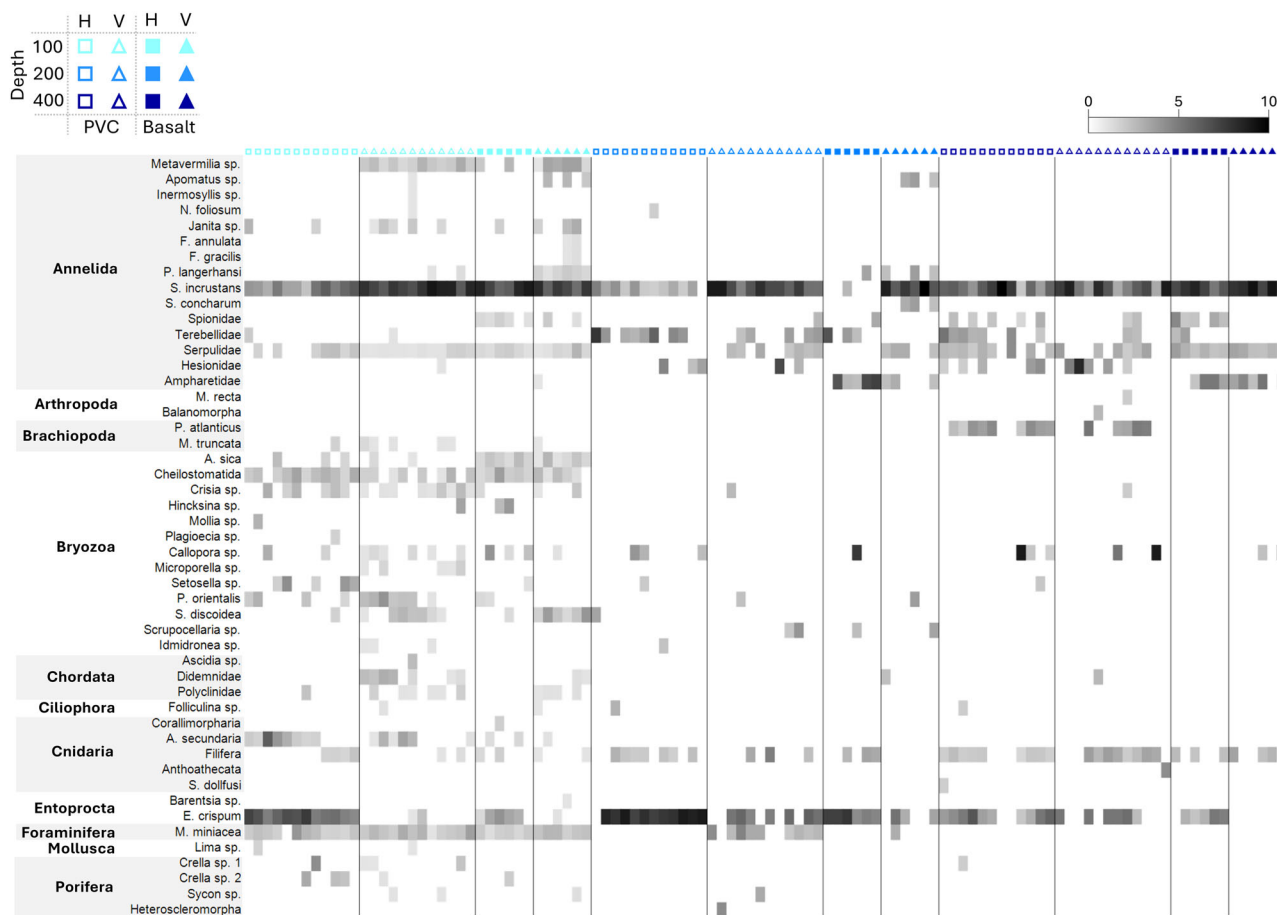
Fig. 1 | Taxa contributions across depths and factors. Shade plot of taxa contribution (standardized and square root transformed abundance data) for the three depths and each considered factors: substrate orientation (horizontal and vertical) and material (PVC and Basalt). Grayscale intensity shows the contribution scale for each taxon expressed on fourth-root transformation data to reduce contributions to similarity by the numerically dominant species. Numerical source data provided in Supplementary Data 1.
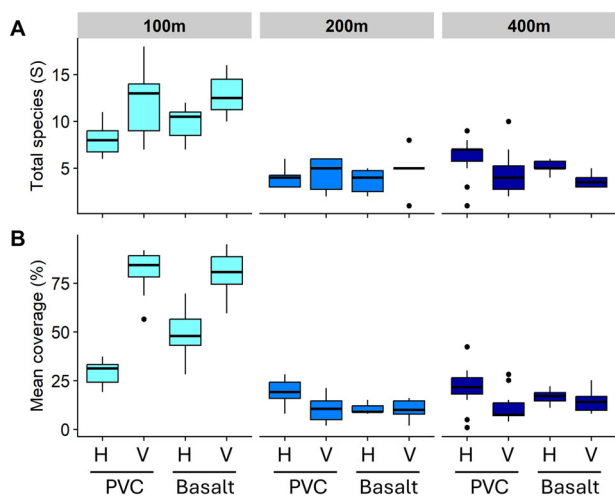
Fig. 2 | Macrofouling species richness and coverage by depth and factors. Median macrofouling (A) species richness and (B) median mean coverage (%) at each depth and for substrate orientation (horizontal, H and vertical, V) and type of material (PVC and Basalt). Numerical source data provided in Supplementary Data 1.

Table 1 | Distance-based linear model marginal tests and overall best solution of the three categorical experimental design factors (transformed in numerals and normalized) as explanatory co-variables (depth, orientation and substrate material) of community structure ordination (based on Bray-Curtis similarity matrix)

Co-variable	SS	Pseudo-F	P
Depth	31304	18.431	0.001
Orientation	29747	17.365	0.001
Substrate material	12460	6.641	0.001
Overall Best Solution	**AICc**	**R^2**	**RSS**
All co-variables	780.76	0.34784	1.3783E + 05

*SS* sum of square, *AICc* Akaike Information Criterion (modified for small sample sizes), *RSS* residual sum of squares.

of the experimental setup (n = 12) (Supplementary Table 4). ANOSIM outputs also corroborate that the type of material had less influence on shaping the structure of macrobenthic communities. Pairwise test results also revealed that most pairwise comparisons were significantly different, except for a few deeper samples (n = 4) (Supplementary Table 4). The influence of the three factors in structuring the macrobenthic communities was also evident when inspecting the patterns in the ordination of the macrofouling assemblages on a mMDS plot of a Bootstrap Average routine (Fig. 4). The plot of the entire dataset showed an array of discrete clusters, particularly evident at 100 and 200 m. The clusters of the 400 m treatments were denser, located closer to each other and slightly overlapped among the two substrate orientations and type of material, suggesting a high degree of similarity within this depth station (Fig. 4).

Fig. 3 | Community structure across depths, orientation, and material. Principal Coordinate Ordination (PCO) plot of macrofouling community structure (based on Bray-Curtis similarity of standardized and square-rooted relative abundance) for the three considered factors (depth, substrate orientation and type of material) and correlations between the three factors (vectors) and PCO axes (blue lines) and between the five DistLM selected taxa (vectors) and PCO axes (red lines). Numerical source data provided in Supplementary Data 1.
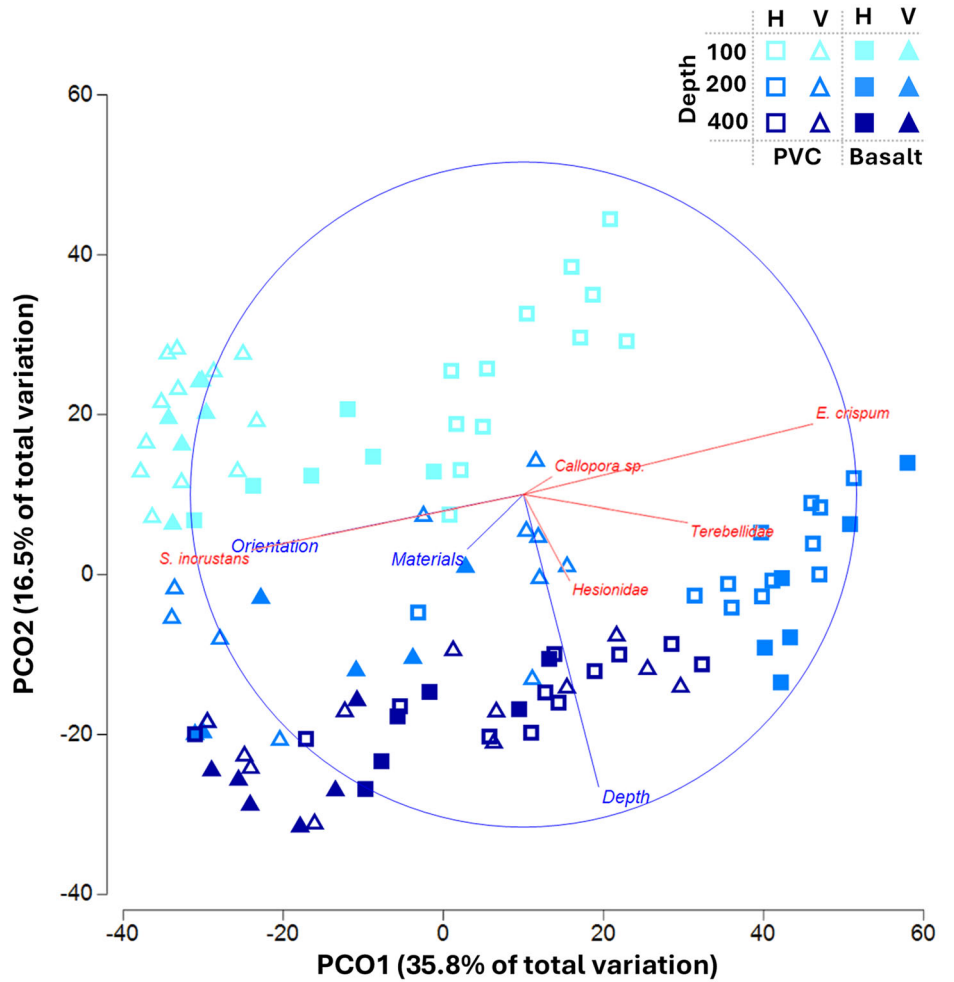
Fig. 4 | Visualization of the level of similarity between macrofouling communities. Metric Multidimensional Scaling (mMDS) plots with Bray-Curtis similarity measure based on the square-root transformation of the macrofouling coverage data. Numerical source data provided in Supplementary Data 1.
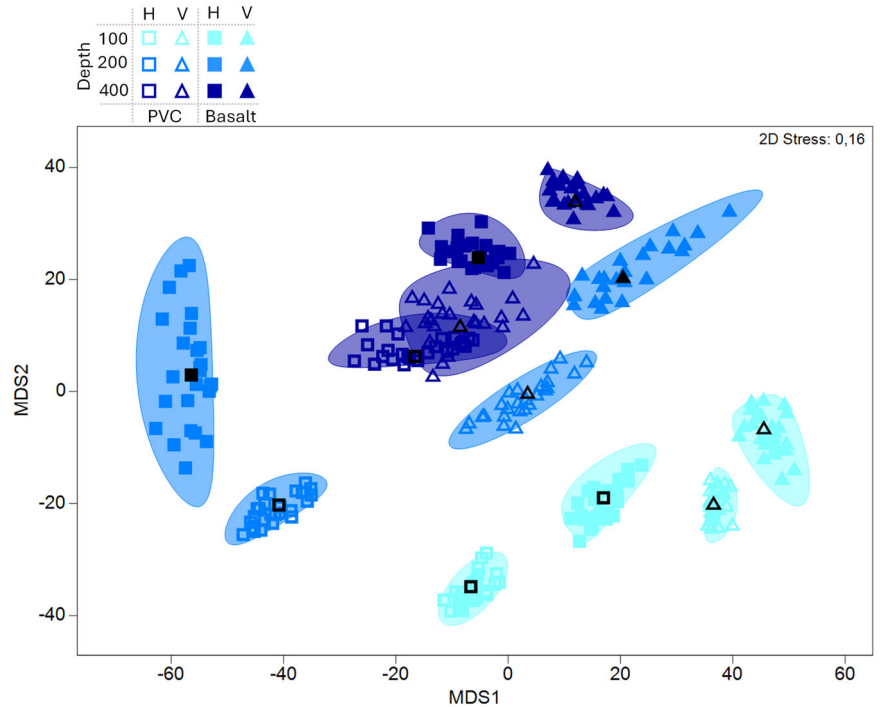
SIMPER analysis was conducted to identify the taxa contributing most significantly to the observed similarity (Supplementary Table 5) in the community structure. The analysis revealed that the most frequent taxa among the top three contributors to similarity were *S. incrustans* (contribution: 19.6–61.5%), *E. crispum* (contribution: 6–57.7%), *Miniacina* cf. *miniacea* (contribution: 11.4–14.8%), Serpulidae (contribution: 10.8–22.7%) and Ampharetidae (contribution: 15.2–29.4%). Conversely, the taxa predominantly responsible for dissimilarity included *S. incrustans* (contribution: 6.3–31.6%), *E. crispum* (contribution: 7.7–31.7%), *Metavermilia sp.* (contribution: 7.0–10.3%), Ampharetidae (contribution: 7.4–17.8%) and Cheilostomatida (contribution: 7.0–10.5%) (Supplementary Data 4).

Discussion

The clear decrease in biodiversity and species abundance observed along the depth gradient considered in this study corroborates the influence of depth in structuring the assemblages of macrobenthic communities, already understood in the marine environment[14,23,52]. This is most likely related to physical factors corresponding to depth, including light, temperature and pressure, that play major roles in shaping the macrobenthic community structure[23,26]. These environmental conditions often restrict the availability of food, which is another crucial controlling factor in the growth and development of macrobenthos organisms, affecting both the diversity and abundance of the species[26]. In our study, after two years of colonization in deep waters, we found at 100, 200 and 400 m depths 41, 23 and 18 taxa respectively. The relatively low biodiversity and species abundance found in the deepest sites of our study are likely influenced by the effects of light attenuation, and it can also be partly explained by surrounding benthic fauna abundance and diversity, and the time needed for visible colonization on hard substrata in this environment[44,45,53].

The most represented taxa along the gradient were the Foraminifera *E. crispum* and the polychaete (Annelida) *S. incrustans*. These two species exhibited a clear preference regarding substrate orientation and, together with Terebellidae, Hesionidae and *Callopora* sp., were the taxa that most contributed to the differences among macrofouling community structures. While *E. crispum* seemed to prefer horizontal surfaces, *S. incrustans* was more abundant and frequent on the vertical plates, suggesting that a plate-orientation effect might be species-specific, as noted in several shallow-water studies[37,54–56].

The abundance and diversity of macrofouling organisms in the vertical plates were higher than in horizontal ones at 100 m, but this apparent influence of substrate orientation on live cover and diversity changed with increasing depth, and at 400 m, the trend seemed the opposite. Similar to our results, a two-year study developed along a depth gradient in the Azores found a more pronounced effect of substrate orientation on macrofouling communities at shallower sites (60 and 150 m) than at the deeper one (500 m) and the up-facing horizontal substrate showed lower species abundance and biodiversity[50]. One possible factor to consider is the effect of siltation and sedimentation of hard substrates[36,56,57]. As with depth, substrate orientation influences other factors, including sediment disturbance[36,56,57]. The impact of gravity deposition of sediments on macrobenthic communities is well known in shallow water, where several studies highlighted that most invertebrates survive better and grow more abundantly on surfaces with a low sedimentation rate[36,57,58]. Multiple coastal development activities and heavy ship traffic in the commercial port of Funchal, located in Madeira's capital, might cause high sedimentation rates, together with water turbidity and pollution, which could explain the vertical orientation preference of macrobenthic communities found in the present study. Considering the natural dispersal of sediments over distance (from shore), this may be an important factor in shaping mesophotic benthic communities[59]. However, further research is required to assess the role of sedimentation in local mesophotic habitats.

In the present study, even if all measured factors played a role in structuring the macrobenthic communities, depth seemed to have a stronger influence, followed by substrate orientation and, finally, type of material. This result agrees with the aforementioned study in the Azores, where the strongest influences came from depth and substrate orientation, while substrate material (PVC vs. limestone) had a weaker effect, influencing only the organisms' accretion[50]. Similar results were obtained in studies on microbenthos communities (i.e. biofilm) grown on plates of different orientations and materials (i.e. titanium, aluminum, limestone, shale and glass) along a vertical depth gradient (i.e. 1500, 2500, 3500 and 4500 m)[44,45]. Again, depth and substrate orientation had a stronger influence in structuring the microbenthos communities, while the type of material played a minor role in the community composition[45]. This pattern was clearly visualized through a similarity assessment (mMDS; Fig. 4), where the entire abundance dataset showed an array of a discrete cluster, particularly evident at 100 m. The discrete cluster in vertical and horizontal substrates indicated a distinct orientation preference at this depth, which was maintained at 200 m, even if less pronounced. However, with increasing depth, the clusters showed a slight overlap for the substrate orientation and type of material, indicating that these factors influenced only moderately the structuring of the macrobenthic communities in the deepest site. Depth and substrate orientation probably had the strongest effect due to their nature as composite factors reflecting the impact of other variables such as light, temperature, pressure, sediment, and abundance of nutrients, predators and larvae[23,24,60].

While water depth and substrate orientation seemed to strongly influence the structure of macrobenthic communities, only slight effects were found for the type of material without a clear trend among treatments. This weak influence of substrate is in agreement with findings of previous shallow-water studies that found that the type of colonizing material seems to have a relatively minor effect on macrobenthos assemblages composition, being its effect restricted to some taxonomic groups[54,55]. Nevertheless, it is worth noting that our findings suggest that some taxa may exclusively grow in artificial PVC substrate, while others only on natural (basalt) substrate. This finding aligns with observations from other studies on artificial substrates, which typically provide conditions that favor the establishment and proliferation of certain species, also including non-indigenous species (NIS)[61,62], and it may be particularly significant when considering the impact of increasing seafloor plastic pollution. Our findings suggest that increasing plastic litter and plastic-based infrastructure on the ocean floor can effectively facilitate the growth and spread of some taxa while hampering other taxa (which would naturally occur in rocky substrates). Further research is recommended to explore the long-term ecological impacts of plastic substrates on macrobenthic community structures, especially concerning the spread of NIS and changes in ecosystem dynamics.

The contribution of different factors to community structure remains poorly understood[63,64], and more so in the mesophotic due to the challenges in performing in situ experiments and the limited knowledge available. Our results provided the first observation of microhabitat preferences in mesophotic macrobenthic communities, showing that their composition was largely influenced by depth and substrate orientation rather than by type of material. In addition to the abiotic factors investigated in the present studies, biotic factors such as predation, life cycle dynamics (e.g., timing necessary for a species population to grow and reproduce), and competition also play significant roles in shaping community structure[63].

These outputs are of primary importance for designing and planning future mesophotic restoration and requalification operations, highlighting the importance of combining different substrate orientations to favor colonization by as many taxa as possible and reducing the effort, focusing on only one type of material. However, additional sites and depths around the archipelago should be considered to capture higher biodiversity, improve our understanding of the mysteries still related to the deep sea and create efficient mesophotic management and conservation programs and restoration operations.

Recruitment experiments in the mesophotic areas are vital for understanding ecosystem dynamics, selecting suitable species, evaluating restoration techniques, monitoring progress and informing conservation efforts and policies. To the best of our knowledge, this experiment represents a novel study in the Madeira archipelago and one of the few studies in the

Fig. 5 | Design and deployment of experimental settlement structures. Design of the experimental settlement structures (50 × 30 × 15 cm) at different depths: (**A**) schematic design, (**B**) picture of the structures after deployment (October 2019) at 400 m depth, (**C**) lateral view, and (D) bottom view. Each structure was equipped with 12 settling plates (14 × 14 cm; PVC or basalt), arranged vertically and horizontally.
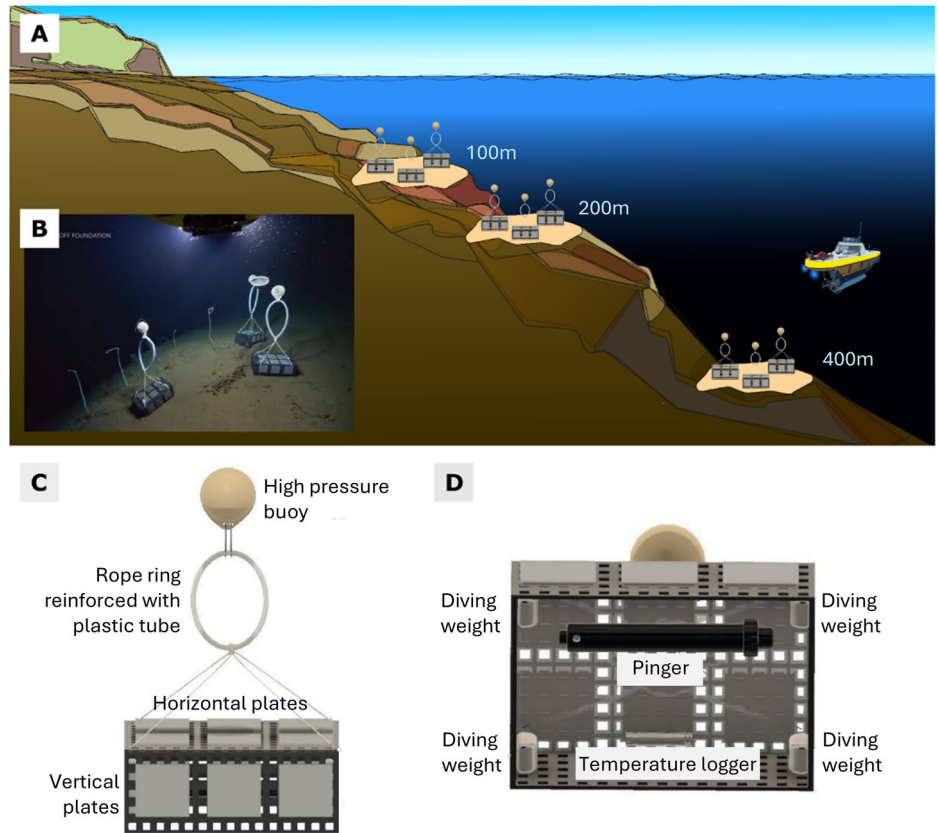
Atlantic Ocean[43,49,50] exploring colonization and fouling on different materials (basalt vs PVC) with different orientations (vertical vs horizontal) at these depths[23,26].

In this context, our findings contribute valuable insights that can shape future mesophotic restoration initiatives and help mitigate the impacts of human activities in this fragile and understudied environment.

Methods

Study site

The Madeira archipelago is part of the Macaronesia region, which includes the Azores, Canary Islands, and Cabo Verde. Occupying only 5.4% of the Macaronesian landmass, the Madeira archipelago is a cluster of Portuguese volcanic islands located in the North-East Atlantic (from 32°23'N to 33°07'N and from 16°15'W to 17°15'W), approximately 900 km southwest of Portugal and about 700 km west of the Moroccan coast. It comprises two inhabited islands (Madeira and Porto Santo) and several smaller uninhabited islands and islets (Desertas and Selvagens). Madeira Island is the largest island of the archipelago, having a surface area of 739 km² (maximum length 58 km and maximum width 23 km).

The Madeira archipelago is surrounded by oligotrophic waters and its bottom topography is characterized by a narrow continental shelf. This topography means that great depths reach very close to the coast, with steep submarine canyons and steep slopes from the abyssal plain (average 3000 to 4000 m deep) almost to the surface[65]. The southern Madeiran coasts reach 1000 m in depth, approximately 1.5 km from the coastline, and at 8 km from the coast average depths are around 2000 m[65].

Experimental design

The experiment of euphotic lower boundary (100 and 200 m) and mesophotic (400 m) zones' macrofouling organisms was conducted off the Bay of Funchal on the south coast of Madeira Island (Supplementary Fig. 2), employing the Human Occupied Vehicle (HOV) LULA1000 (maximum depth of 1000 m with a three-person crew), operated by the Rebikoff-Niggeler Foundation and supported by the ADA REBIKOFF vessel. This vessel was specifically designed to lift, transport and deploy the submersible, and it is equipped with underwater navigation and communication systems to record near real-time geographical positions and depth of the submersible (see[14] for more details of LULA1000 instrumentation and associated equipment). Using a submersible offers multiple advantages over shipboard operations, including characterizing the environment, selecting flat, stable deployment sites and allowing careful placement of the experimental structures on the seafloor. An HOV allowed us to overcome technical issues in performing in situ deep-sea experiments and was indispensable to bringing and retrieving the experimental structures that hosted the colonization plates to study the mesophotic macrofouling communities.

The experimental structures (Fig. 5) were built using resistant plastic boxes (50 × 30 × 15 cm) equipped with 8 kg on the box base to keep them well-fixed to the bottom. To each structure, 12 polyvinylchloride (PVC, 14 × 14 × 0.3 cm) or basalt (14 × 14 × 3 cm) settling plates were attached, six vertically (V) and six horizontally (H) orientated (Fig. 5). The PVC settlement plates were sanded with fine sandpaper (P120) to homogenize and remove the shine from the surface plate and facilitate organisms' settlement. A high-pressure buoy was attached to each box to support a rope ring reinforced with a plastic tube, necessary for docking and transport with the submersible (Fig. 5). One of the structures for each depth was equipped with a temperature logger to monitor the temperature fluctuation during the experiment (Supplementary Fig. 1, Supplementary Data 2) and a long-time acoustic pinger to facilitate the retrieval at the end of the experiment (Fig. 5).

Sampling

At the beginning of October 2019 (8th October 2019), the experimental structures were deployed at 100, 200 and 400 m depths on a soft sediment and relatively flat area to assess macrofouling colonization at different depths. Two structures hosting PVC plates and one hosting basalt plates were deployed at each depth for a total of nine experimental structures. The experimental structures were left at each of the three depths for almost two years (~24 months).

On the date of retrieval (24th September 2021), all structures were still located in the same position and were retrieved with the submersible LULA1000. On this occasion, the submersible LULA1000 was equipped with a custom-made collection device that transported all three structures to the surface simultaneously from each depth.

The settling plates, all hosting macrofouling organisms, were detached from the structures, sorted into labeled plastic bags containing seawater and transferred to the laboratory for further analysis. Each plate was photographed with an Olympus (Tough TG-6) camera and was carefully examined with a stereomicroscope (Leica S8APO) to quantify the total species pool and identify the fouling organisms to the lowest possible taxonomic rank. Unknown or dubious specimens were photographed at the maximum detail, collected, and preserved in 95% ethanol (according to morphotype/taxa) for later species determination by specialized taxonomists. Damaged or ambiguous specimens were only identified at higher taxonomic levels (Order, Family, or genus) to avoid misidentification.

The community composition was determined for each settling plate, including the species richness and percent cover of each species, biofilm (unidentified organic material aggregated), bare space and sediment (acknowledging that some sediment was dissipated during the retrieval of the structures, we recorded any remaining sediment on the plates during the analysis). Using the image analysis software Coral Point Count with Excel extension (CPCe)[66], each image was subdivided into 3 x 3 grids of 9 cells, with 11 random points per cell, resulting in 99 points analyzed per picture. This stratified random sampling method, successfully used in previous studies (e.g.[27,30]), ensured that points were sampled in each image region.

Temperature recordings

Attached to one structure of each depth, a temperature sensor (Star-Oddi) was programmed to take measurements every six hours ($n = 4$/day). Average and extreme monthly temperatures (Mean ± SE and min-max) were calculated for each depth. Since temperature is related to depth[23,24] and was clearly distinct at the three considered depths (Supplementary Fig. 1), we used depth as a factor and co-variable that may shape differences in the fouling community.

Statistics and reproducibility

CPCe data point annotations were used to estimate the relative abundance of each taxon on each settlement plate and to assess the community structure. Biofilm and on-living categories (bare, sediments) were excluded. All data (i.e. macrofouling taxa classified to the lowest taxonomic category possible) was standardized, fourth-root transformed (to reduce the weight of dominant taxa), and a Bray-Curtis similarity matrix was constructed for further analysis.

Since the number of structures was heterogeneous (two structures with PVC and one with basalt), to compare the communities' attributes between PVC and basalt plates, a pairwise PERMANOVA test based on Bray-Curtis similarity was initially performed to test differences among the three structures at each depth. Since no significant differences were found between the two structures hosting PVC plates at each depth, subsequent PERMANOVA analyses were performed considering only one set of PVC plates randomly selected to have a balanced dataset between PVC and basalt. An analysis of Similarity (ANOSIM) was used to assess variation in taxa composition across the 12 possible combinations of factors (i.e. depth, material and orientation). The mean coverage heatmap was created with matrix display in PRIMER v7 plus and based on the taxonomy data previously obtained to visualize the most dominant taxa at phylum and genus/species levels.

To assess the relationship and contribution of each factor in shaping the community structure, we conducted a Distance-based Linear Model (DistLM) analysis Akaike Information Criterion (AICc) and a Best selection procedure with 999 permutations to identify the top three solutions with the highest contribution to explain samples ordination[67]. DistLM marginal tests were conducted to assess significant correlations ($p < 0.05$) of each individual factor with Bray-Curtis similarity of macrofouling assemblages. In these tests, categorical factors were converted to numerical values and normalized as follows: Depth ("1" for 100 m, "2" for 200 m, and "3" for 300 m), Plate orientation ("1" for horizontal and "2" for vertical), and Substrate type ("1" for PVC and "2" for basalt). Additionally, the selection procedure outputs were used to identify the best explanatory solution by considering all possible combinations of these three factors.

Similarities in the structure of macrofouling communities and the role of different factors such as depth, substrate orientation and type of material were assessed by inspecting a Principal Coordinate Ordination (PCO) plot and a metric Multidimensional Scaling (mMDS) plot with bootstrap averages[71]. Where significant differences were observed among groups, the contribution of each taxon to the similarity/dissimilarity within/between community groups was further investigated with similarity percentage analysis (SIMPER)[67].

All statistical analyses of macrofouling community attributes (number of species, per cent coverage and community structure) were conducted using the software package PRIMER v7 with PERMANOVA.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

All data generated or analyzed during this study are included in this published article (and its Supplementary Information files). Source data for all the figures and plots in the manuscript can be found in Supplementary Data 1.

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