

# Variations in epilithic microbial biofilm composition and recruitment of a canopy-forming alga between pristine and urban rocky shores

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## ABSTRACT

Brown algae of the genus *Ericaria* are habitat formers on Mediterranean rocky shores supporting marine biodiversity and ecosystem functioning. Their population decline has prompted attempts for restoration of threatened populations. Although epilithic microbial biofilms (EMBs) are determinant for macroalgal settlement, their role in regulating the recovery of populations through the recruitment of new thalli is yet to be explored. In this study, we assessed variations in microbial biofilms composition on the settlement of *Ericaria amentacea* at sites exposed to different human pressures. Artificial fouling surfaces were deployed in two areas at each of three study sites in the Ligurian Sea (Capraia Island, Secche della Meloria and the mainland coast of Livorno), to allow bacterial biofilm colonization. In the laboratory, zygotes of *E. amentacea* were released on these surfaces to evaluate the survival of germings. The EMB's composition was assessed through DNA metabarcoding analysis, which revealed a difference between the EMB of Capraia Island and that of Livorno. Fouling surfaces from Capraia Island had higher rates of zygote settlement than the other two sites. This suggests that different environmental conditions can influence the EMB composition on substrata, possibly influencing algal settlement rate. Assessing the suitability of rocky substrata for *E. amentacea* settlement is crucial for successful restoration.

## 1. Introduction

In marine environments, canopy-forming brown macroalgae such as kelps (Laminariales) and fucaceans (Fucales), along with mussels, corals and seagrasses, play a key role as habitat formers, sustaining biodiversity and ecosystem functioning (Angelini et al., 2011; Bertocci et al., 2015; Bruno, 2000; Gribben et al., 2019; Thomsen et al., 2010, 2022) and providing important ecosystem services, including coastal protection, carbon sequestration and nutrient cycling (Costanza et al., 2014; Howard et al., 2017; Macreadie et al., 2019; McLeod et al., 2011; Orth et al., 2006). Seagrasses and canopy-forming macroalgae are highly sensitive to extreme climatic events (e.g. marine heatwaves, storms) and anthropogenic disturbances (de Bettignies et al., 2018; Wernberg et al., 2016). A generalized decline of populations of these organisms has been reported globally (Airoldi and Beck, 2007; Mangialajo et al., 2008; Monserrat et al., 2022).

Brown algae belonging to the complex *Cystoseira sensu lato* (genera *Cystoseira* C. Agardh, *Ericaria* Stackhouse and *Gongolaria* Boehmer) form intertidal and shallow subtidal forests along Mediterranean rocky shores (Benedetti-Cecchi et al., 2001; Bulleri et al., 2002) that are considered among the most diverse and productive coastal habitats (Duarte et al., 2022; Pessarrodona et al., 2022). Nonetheless, these communities are currently threatened by anthropogenic pressures, such as over-exploitation of predatory species, urbanization, eutrophication and climate change. Indeed, loss or marked declines of macroalgal forests have been documented throughout the Mediterranean basin (Arévalo et al., 2007; Bellard et al., 2012; Benedetti-Cecchi et al., 2001; Orfanidis et al., 2021; Strain et al., 2014).

Such decline has prompted conservation strategies, such as the establishment of Marine Protected Areas (MPA) (Solan et al., 2020) or the reduction of non-climatic pressures (pollution, overfishing) (Gattuso et al., 2018). However, due to the low dispersal of zygotes of most

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*Cystoseira s.l.* species (i.e., over a few tens of cm) (Clayton, 1992; Reynes et al., 2021; Riquet et al., 2021), natural recovery is very unlikely in areas distant from extant populations. This has raised interest towards active restoration strategies that include either the transplantation of individuals from healthy populations (Falace et al., 2006; Susini et al., 2007) or non-destructive methods aimed to enhance recruitment, via *in situ* techniques, consisting in positioning fertile receptacles in the field, and *ex situ* techniques, consisting in the outplant of juveniles produced from fertile receptacles in the laboratory (Medrano et al., 2020; Verdura et al., 2018).

The ability of restored thalli to survive and successfully reproduce and, hence, to self-maintain and/or expand the local population underpins the success of restoration activities. Within this context, the role of epilithic microbial biofilm (EMB) in regulating population recovery through the recruitment of new individuals is yet to be explored, despite they have been shown to be key determinants for recruitment of benthic species (Hadfield, 2011; Keough and Raimondi, 1996; Park et al., 2011; Wahl et al., 2012; Zhou et al., 2016), including macroalgae (Mieszkin et al., 2013). Biofilms form on all hard substrata and are agglomerates of macromolecules, prokaryotic and eukaryotic microorganisms (Caruso, 2020). Epilithic microbial biofilms are important functional components of the coastal ecosystem as primary producers, promoters of larval settlement, and food resources for herbivorous animals (Keough and Raimondi, 1996; Kim et al., 2014; Yallop et al., 1994). The composition of EMB is influenced by physical conditions, such as temperature, nutrient concentration, sedimentation rates, light levels and wave-exposure (Antunes et al., 2019; Cacabelos et al., 2020; Dang and Lovell, 2016), but also by extant benthic organisms. For instance, macroalgae can condition EMB through shading, abrasion and exudation of dissolved organic carbon (DOC) and allelochemicals (Box and Mumby, 2007; Egan et al., 2013; Nugues et al., 2004; Nyström et al., 2012; Rasher and Hay, 2010; Vega Thurber et al., 2012). Due to large variations in the production and release of DOC and allelochemicals, assemblages dominated by different macroalgal species can be expected to host different EMBs (Haas et al., 2011). Recent studies have, indeed, shown that the structure of EMBs differs among habitats, namely macroalgal canopies, urchin barrens and algal turfs, that compose mosaics on Mediterranean rocky reefs (Elsherbini et al., 2023).

In this study, using artificial fouling surfaces, we tested the hypotheses i) that the composition of the EMB would vary among rocky substrata from sites exposed to different human pressures (i.e., pristine versus urban) and dominated by different macroalgal species (i.e., canopy-forming versus turf-forming), and ii) that recruitment rates of the fucal seaweed *Ericaria amentacea* (C. Agardh) Molinari & Guiry will vary between pristine and urban sites.

## 2. Materials and methods

### 2.1. Study site

This study was performed between April and August 2021 at three sites in the NW Mediterranean: Capraia Island (43°03'09.8"N, 9°50'16.0"E), the coast of Livorno (43°31'46.6"N, 10°18'12.8"E) and the MPA of Secche della Meloria (43°32'47.2"N, 10°13'15.5"E) (Fig. 1). Capraia Island is located about 30 miles off the mainland coast of Tuscany, in the Northern Tyrrhenian Sea. This island is included in the Tuscan Archipelago National Park and can be considered pristine due to very limited urban development and lack of major industrial activities (Tamburello et al., 2012). Rocky shores around the island are characterized by the presence of macroalgal canopies formed by *Ericaria amentacea* and *E. brachycarpa* (J. Agardh) Molinari & Guiry, in low intertidal (between 0.2 m and -0.5 m above/below mean water level) and shallow subtidal habitats (1–15 m depth), respectively. Reefs at the MPA of Secche della Meloria and along the coastline of the city of Livorno, that are considered as urban sites, are exposed to inputs of sediments, nutrients, organic and inorganic pollutants (Tamburello

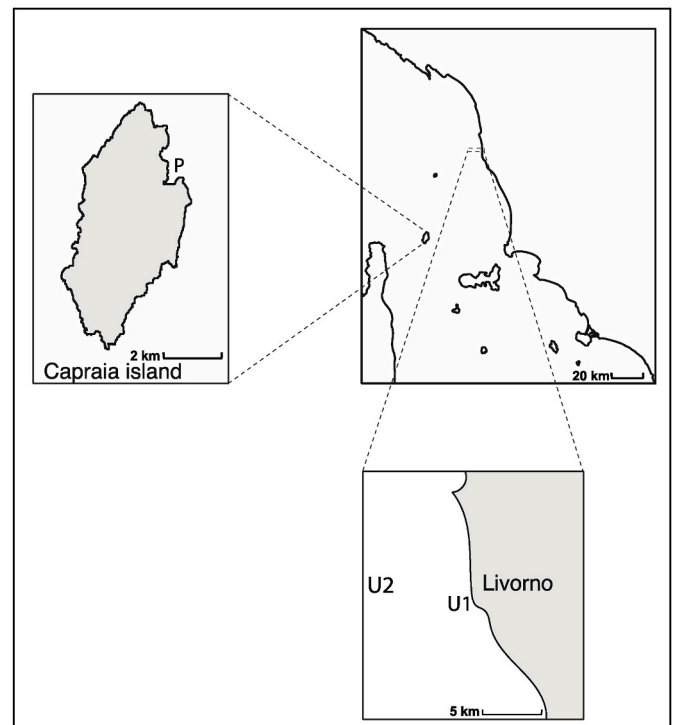


Fig. 1. Map showing the study sites. Pristine site: Capraia Island; Urban site 1: Livorno (U1); Urban site 2: Secche della Meloria (U2).

et al., 2012) and are characterized by the presence of turf-forming macroalgal assemblages a few cm thick that are mainly composed by filamentous species (e.g., *Ceramium* spp., *Sphacelaria* spp.), articulated coralline forms (e.g., *Ellisolandia elongata* (J.Ellis & Solander) K.R. Hind & G.W. Sanders, *Jania rubens* (Linnaeus) J.V. Lamouroux) and coarsely branched algae (e.g., *Chondria* spp. and *Laurencia* spp.) (Bulleri et al., 2011; Bulleri and Benedetti-Cecchi, 2006; Tamburello et al., 2015).

Seawater temperature and pH at Capraia island and Livorno were monitored using HOBO data loggers in summer 2021. In July, the mean temperature varied between 25.8 °C in Capraia island and 24.8 °C in Livorno. The average pH ( $\pm$ SE) at Capraia island was  $8.08 \pm 0.01$ , while that at the urban site was  $7.97 \pm 0.05$ .

The study site at Capraia and that on the coast of Livorno were characterized by different nutrients concentrations. Data collected from previous studies at the same sites (June 2015) showed that dissolved inorganic nitrogen (DIN) concentrations ( $\mu\text{mol/L}$  nitrates and nitrites) were  $0.29 \pm 0.06$  and  $0.40 \pm 0.02$  for the pristine and urban site respectively, while phosphate concentration ( $\mu\text{mol/L}$ ) was  $0.09 \pm 0.01$  at the pristine site and  $0.12 \pm 0.004$  at the urban site. Likewise, in November 2015, DIN and phosphate concentrations were  $0.24 \pm 0.02$  and  $0.04 \pm 0.004$ , respectively, at the pristine site and  $0.72 \pm 0.1$  and  $0.25 \pm 0.04$ , respectively, at the urban site.

In order to compare variation in macroalgal settlement among substrata colonized by different EMBs, artificial fouling surfaces, previously rinsed with sterile water and scratched with sandpaper, were deployed at the pristine site (Capraia Island) and at both urban sites (Livorno and Secche della Meloria). Unbiased assessment of the effects of the EMB on macroalgal recruitment required artificial substrata to be free from multicellular colonizers, such as invertebrates or macroalgae. Thus, it was necessary to synchronize the development of the EMB on artificial tiles with the availability of mature receptacles for production of germlings in the laboratory. Our study was initially designed to use *E. brachycarpa* as model species and a first batch of artificial surfaces was deployed at depths between 2 m and 5 m in April 2021. In particular, at each study site, four plexiglas panels, each supporting six clay tiles (5 × 5 cm each), were fixed about 10 m apart from each other, in each of two

areas separated by about 1 km. Panels were attached to the substratum using stainless-steel screws.

Starting in April 2021, the reproductive status of the two species of *Ericaria* was monitored at Capraia Island. Every ten days, ten thalli of different individuals of both species were collected from the field and transported to the laboratory, where apices bearing visible receptacles (the reproductive structures, see Cormaci et al., 2012) were observed with an optical microscope. Cross sections of some receptacles were cut with a razor blade in order to assess the maturity of the receptacles (i.e., whether mature oogonia were present or not within the receptacles). Since we did not observe fertile receptacles until July, when tiles appeared colonized by macroalgae and invertebrates, tiles deployed in April were replaced in July with a new batch of panels that supported new tiles.

In July 2021, *E. amentacea* developed mature receptacles, which, instead, were not observed in *E. brachycarpa* throughout the entire period of monitoring (April to November 2021). Thus, *E. amentacea* was used for the settlement trials. Since tiles were deployed in the shallow subtidal zone, the EMB used for the settlement trial might be not representative of those that develop in mid or low intertidal habitats (i.e., up to 1 m depth). For this reason, our experiment should be considered as a test of the effects of different EMBs on the settlement of *E. amentacea* and differences between the pristine and the urban sites should be interpreted with caution.

After three weeks, a time sufficient to allow colonization by bacterial biofilms (L. Pedicini personal observations), tiles were collected and transported to the laboratory, using an icebox filled with seawater, where 7 tiles from each of the two areas within each site were placed in each of two 5 L filtered seawater tanks. The temperature and photoperiod in tanks were controlled to reflect the conditions at the sampling sites during the reproductive phase of *E. amentacea*. The light intensity was provided by LED lamps and set at  $125 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  with a photoperiod of 15:9 h light:dark. The temperature was set at 26 °C and checked with Onset Hobo data loggers.

In the meanwhile, healthy apices bearing mature receptacles of different individuals of *E. amentacea* were collected from the low-shore zone at Capraia Island. Apices, wrapped in seawater-wetted towels, were transported to the laboratory and stored at 4 °C in the dark for 24 h to stimulate the gamete release (Falace et al., 2018). Three randomly chosen apices with mature receptacles were rinsed with sterile seawater to remove detritus and then placed over 4 tiles in each tank, with the same conditions mentioned above. The remaining tiles were used for the characterization of the biofilm. Two glass slides were placed in each tank in order to monitor the zygote development using a binocular microscope with 40× magnification. The culture medium was renewed every 4 days to minimize the effect of nutrient limitation and was continuously aerated by air pumps.

One week after the collection of the tiles from the field, the germlings were visible to the unaided eye on each tile and the apices were removed. Each tile was photographed under a stereo-microscope (Fig. 2) and the number of germlings was counted in 5 quadrats (1 × 1 cm each) selected at random in each tile. The count was performed by processing

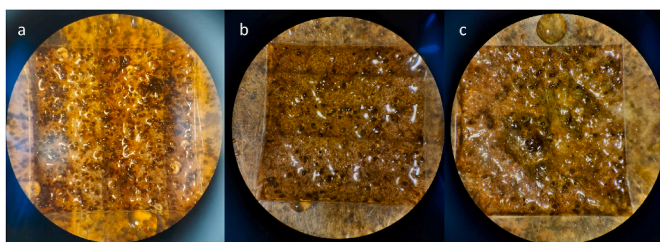


Fig. 2. *E. amentacea* germlings on tiles collected from Capraia Island (a), Livorno (b) and Secche della Meloria (c), photographed two weeks after the release of zygotes.

photographic data using the free software ImageJ (Rueden et al., 2017). The counts were repeated after two weeks since the start of the experiment, once non-attached zygotes/germlings were removed by gentle water washing to obtain an unbiased estimate of settlement.

## 2.2. Analysis of metabarcoding data

The microbial communities on 3 tiles from each tank, after a week of adaptation to laboratory conditions, were sampled with sterile cotton tips, which were used to gently swab the tile surface for 30 s (Marzinelli et al., 2018). Each swab was then immediately transferred into a cryogenic tube and stored at -20 °C until DNA extraction. Total DNA from the samples was extracted from each cotton tip using the QIAGEN DNeasy® PowerSoil® Pro kit following the manufacturer's instructions and DNA quantity was checked using a Qubit Fluorometer (Table S1 in Supplementary material). Subsequent tgDNA PCR-amplification and purification of the V3–V4 hypervariable regions of the 16s rRNA gene were conducted at BMR genomics (Padua, Italy), using the universal bacterial primer pair Pro341 F: 5'-CCTACGGGNGCASCAG -3'/Pro805 R: Rev 5' GACTACNVGGGTATCTAATCC -3' (Takahashi et al., 2014). Sequencing was performed on an Illumina MiSeq platform using a 2 × 300 bp paired-end approach.

The obtained sequences (raw reads) were deposited in the European Nucleotide Archive (ENA) under the study accession number PRJEB60263. Reads were demultiplexed and sequences were quality trimmed using QIIME2 (Bolyen et al., 2019). Reads were truncated at 273 bp length (forward reads) and 269 bp (reverse reads). Then, quality filtering, primer trimming, pair-end read merging and de novo chimera removal were performed with DADA2 (Callahan et al., 2016). Sequences were grouped, using DADA2, in amplicon sequence variants (ASVs). For the taxonomic assignment, a naive-Bayes classifier was trained on the SILVA v132 database (Quast et al., 2013), built with 99% similarity clustered Operational Taxonomic Unit. ASVs identified as chloroplast or mitochondria were excluded from further analyses.

## 2.3. Statistical analyses

DNA extraction and sequencing were successful for 4 tiles from Capraia Island and 7 tiles from Livorno, but only for two replicates from Secche della Meloria, which were, therefore, excluded from the analysis. Differences in the bacterial community structure were compared using the Bray-Curtis coefficient with untransformed relative abundances of ASVs from the rarefied dataset. Variation among sites in the bacterial community was assessed by means of two-way PERMANOVA (Anderson, 2001) including the factors Site (2 levels, fixed) and Area (2 levels, random and nested within Site). For this response variable, the factor Tank was not included in the analysis due to the loss of both tiles of two tanks for Capraia Island. When the low number of unique permutations did not allow to make inference at the established significance level ( $p < 0.05$ ), the Monte Carlo procedure was used to calculate  $p$ -values (Anderson, 2017). Non-metric multidimensional scaling (nMDS) was used to visualize multivariate patterns.

Families contributing most to differences in the bacterial composition between the pristine and the urban site were calculated using Similarity Percentage (SIMPER) analysis.

Differential ASVs between sites were identified using the negative binomial GLMs. ASVs with adjusted  $p$  values  $< 0.01$  were investigated through pairwise comparisons between pristine and urban sites. The R (version 4.2.0) packages “phyloseq”, “vegan” (Oksanen et al., 2022), “DESeq2” (Love et al., 2014), “tidyr” (Wickham et al., 2019) and “dplyr” (Wickham et al., 2018) were used for statistical analyses.

Average beta-diversity among tiles within each area was computed using the two areas for each site as replicates. We measured beta-diversity as the average distance from a sample to the group centroid, based on Bray-Curtis dissimilarity matrix (Anderson et al., 2006), using the function “betadisper” of R package “vegan”. Differences between the

pristine and the urban site were assessed by means of one-way ANOVA including the factor Site (2 levels, fixed). The assumption of homogeneity of variances was tested by Cochran's C-test.

Alpha-diversity was estimated as the number of ASVs and the Shannon index, using the package "vegan". Since the design did not have a balanced number of replicates for each area, differences in alpha-diversity between sites were analyzed with a Generalized Linear Mixed Model with gaussian distribution (using the R "glmmTMB" package) (Brooks et al., 2017) including Site as the fixed factor and Area in the random part of the model. Model assumptions were checked with the R package DHARMA (Hartig and Lohse, 2022).

Variations in the density (number per cm<sup>2</sup>) of settled germlings between the pristine site and urban sites were assessed by means of asymmetrical analysis of variance (ANOVA) (Underwood, 1992, 1994). This analysis included the factor Site (fixed), Area (random and nested within Site) and Tank (random and nested within Area and Site). The Site term was partitioned into the contrast "pristine versus urbans" and the "among urban sites" source of variation. Cochran's C-test was used to check the assumption of homogeneity of variances. The analysis was repeated separately for each sampling date using the R package GAD (Sandrini-Neto and Camargo, 2012).

### 3. Results

#### 3.1. Differences in bacterial communities among pristine and urban sites

*Bacteroidetes* and *Protobacteria* were the most abundant phyla at both the pristine and the urban site (Fig. S1 in Supplementary material). Likewise, *Alphaproteobacteria*, *Gammaproteobacteria* and *Bacteroidia* were the most abundant classes at both sites (Fig. S2 in Supplementary material).

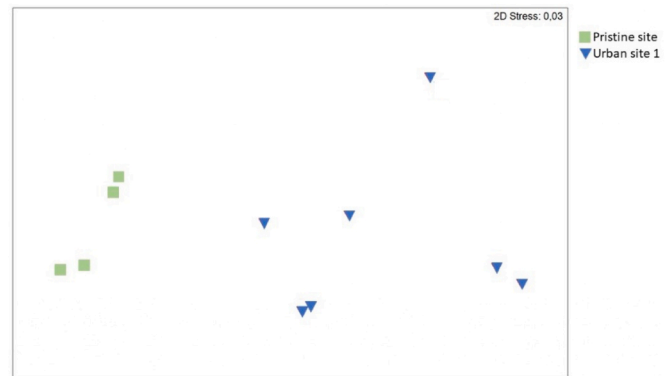
At the family level, the EMBs were dominated by *Vibrionaceae*, *Rhodobacteraceae*, *Flavobacteriaceae* and *Hyphomonadaceae* (Fig. 3). The SIMPER analysis indicated 28 families as those contributing most to differences between the two sites at this level of taxonomic resolution (Table S2 in Supplementary material).

When compared at the ASV level with PERMANOVA, microbial communities on tiles deployed at the pristine site differed from those deployed at the urban site (Table 1 and Fig. 4). There were 65 ASVs, out

**Table 1**

Results of PERMANOVA comparing the structure of bacterial assemblages (identified at ASV level) between sites (Capraia vs. Livorno) and two areas in each site. The significant result is reported in bold.

Sources of variation	Df	MS	Pseudo-F	p (perm)	Unique perms	p (MC)
Site	1	9581.2	3.3219	0.174	12	<b>0.0487</b>
Area (Site)	2	2945.2	1.6479	0.0964	8666	0.1458
Residual	7	1142.7				

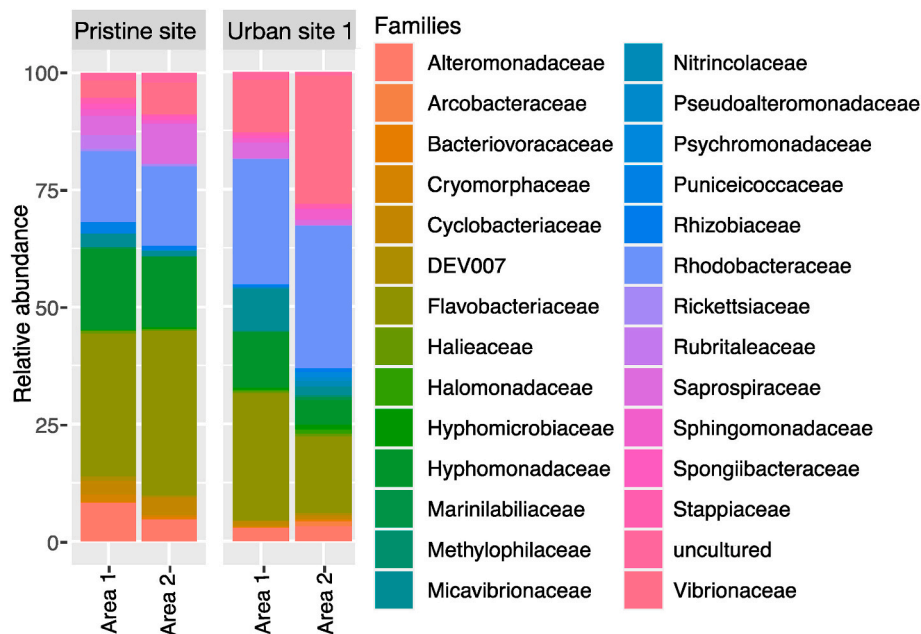


**Fig. 4.** nMDS ordination visualizing differences in bacterial assemblages between the pristine site (Capraia Island) and the urban site 1 (Livorno) at the ASV level.

of total 1021 compared, which varied between sites ( $p < 0.01$ ). ASVs belonging to the families of *Flavobacteriaceae*, *Hyphomonadaceae* and *Alteromonadaceae* had a greater abundance at the pristine site, while ASVs belonging to *Vibrionaceae*, *Rhodobacteraceae* and *Sphingomonadaceae* were more abundant at the urban site (Fig. 5).

Alpha-diversity (both ASV richness and Shannon) of microbial communities did not differ between the pristine and the urban site (Table S3 in Supplementary material).

In contrast, the one-way ANOVA showed that the two sites differed in



**Fig. 3.** Relative abundance of the most abundant bacterial families at the pristine site (Capraia Island) and the urban site 1 (Livorno). Each bar represents an area of the study site.

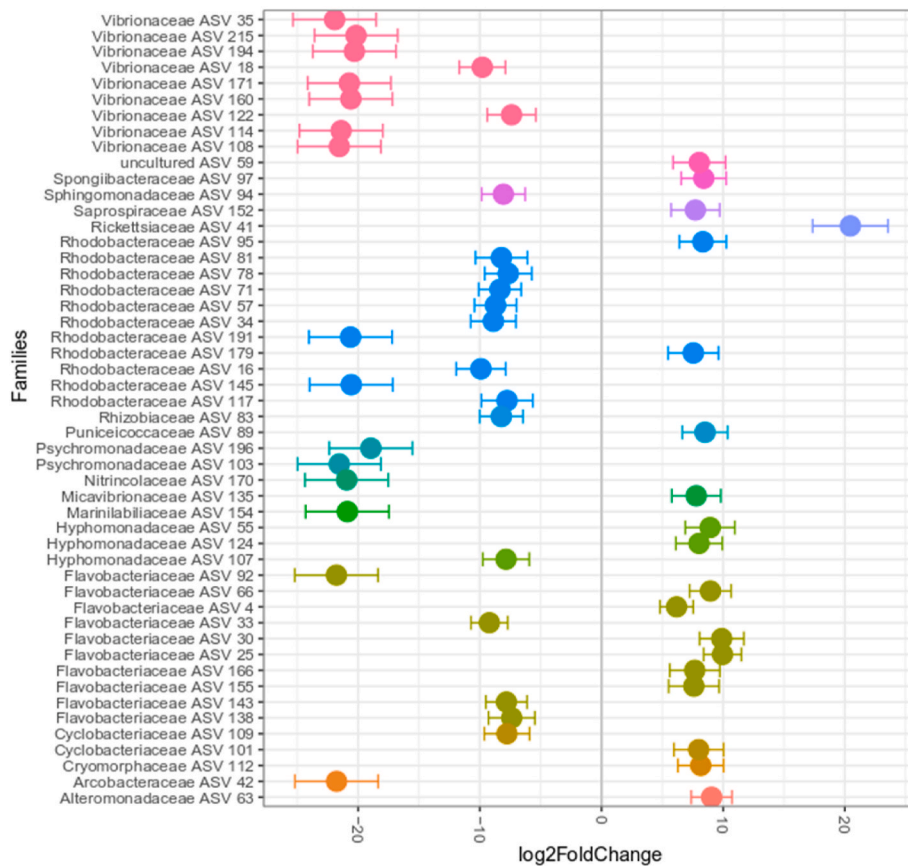


Fig. 5. Log fold change (LFC, Log 2) in abundance of 50 differential Amplicon Sequence Variants (ASVs) between pristine and urban sites (ASVs having higher or lower abundance in the pristine site are represented in the right and left panel, respectively).

terms of beta-diversity (Table S4 in Supplementary material), suggesting that variation between areas (at the scale of 1000 s m) in the bacterial community composition was higher at the urban site than the pristine site.

### 3.2. Differences in *E. amentacea* settlement

There was a significant effect of the contrast Pristine vs Urbans on the number of germlings of *E. amentacea* both after 1 and 2 weeks since zygote release (Table 2). The density of germlings was higher on the tiles from the pristine site compared to the urban sites, which did not differ from each other (Fig. 6), consistently between sampling dates. There was significant variation in the density of germlings among tanks at both sampling times (Table 2).

Table 2

Results of asymmetrical ANOVA on the effects of different sites on the number of germlings of *E. amentacea* after one and two weeks since gamete release. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

Settlement rate of <i>E. amentacea</i>					
Source of variation	df	Week 1		Week 2	
		MS	F	MS	F
Site (S)	2	70805	28.44*	49949	23.60*
Pristine (P) vs Urbans (U)	1	135921.6	54.60**	93101	43.99**
Among urbans	1	5688.9	2.28	6797.78	3.21
Area (Site)	3	2489	0.29	2116	0.17
Tank (Area)	6	8433	2.65*	12139	7.65***
Residual	36	3179		1587	
Transformation		none		none	
Cochran's test		ns		ns	

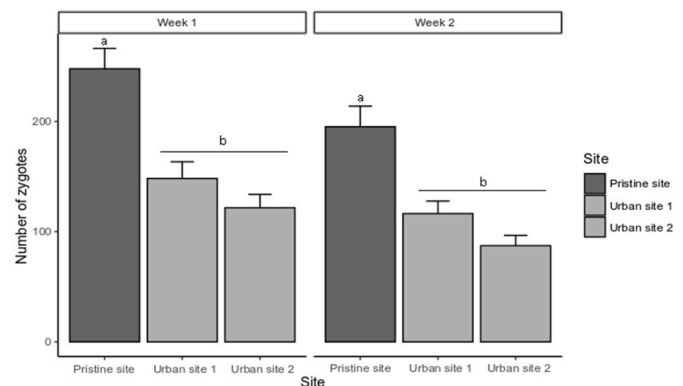


Fig. 6. Number (mean + SE, n = 5) of *E. amentacea* germlings from the three study sites (Pristine = Capraia Island, Urban 1 = Livorno, Urban 2 = Secche della Meloria) at the first and the second sampling date (one and two weeks after gamete release, respectively). Different lowercase letters above bars denote significant differences among sites.

## 4. Discussion

Despite the pristine and the urban sites shared most of the abundant bacterial families, namely *Vibrionaceae*, *Rhodobacteraceae*, *Flavobacteriaceae* and *Hyphomonadaceae*, EMBS differed between these environments when compared at the ASV level. In addition, bacterial communities also differed between the pristine and the urban site in terms of beta-diversity. Such differences may have, in turn, influenced the settlement of *E. amentacea*, which was greater on tiles from Capraia Island than on tiles from the urban sites.

EMBs from Capraia Island were characterized by greater abundances of ASVs belonging to the families *Flavobacteriaceae*, *Hyphomonadaceae* and *Alteromonadaceae*. In contrast, at the urban sites, there was a greater relative abundance of *Rhodobacteraceae*, a family that includes copiotrophic bacteria that generally grow on organic matter-rich substrates and are known to play a key role in algal C cycling (Michael et al., 2016; Ziegler et al., 2016). Unfortunately, technical problems prevented assessing the composition of bacterial biofilms colonizing tiles at the other coastal site (Secche della Meloria). However, the numbers of settled germlings of *E. amentacea* at this site were comparable with those at the other urban site. Indeed, despite being located about 3 miles off-shore and included within an MPA, the Secche della Meloria site is in front of the major commercial port of Livorno and under the influence of the discharging plume of the Arno river and, thus, exposed to enhanced loading of nutrients and high levels of organic and inorganic pollution (Tamburello et al., 2012). In addition, this site is affected by intense boat traffic and anchoring during the summer season (F. Bulleri, personal observation).

The composition of microbial assemblages developed at urban sites may have been directly influenced by local environmental conditions, such as light, water flow, sedimentation rates and water chemistry (Pfister et al., 2019), but also by dominant benthic species. A recent study has documented marked variations among alternative habitats, namely macroalgal canopy stands, algal turfs and urchin barrens, that compose mosaics on rocky reefs (Elsherbini et al., 2023). Several mechanisms could contribute to such differences in EMB composition among habitats, including DOC release rates (Haas et al., 2011) and production of allelochemicals (Harlin and Rice, 1987). Dominance of canopy-forming species belonging to the genus *Ericaria* at the pristine site and of algal turfs at coastal sites, along with variations in environmental conditions (Tamburello et al., 2012), may, thus, underpin the differences in EMB composition.

Bacteria regulate settlement, germination, growth and morphology of many organisms, including macroalgae (Dobretsov and Qian, 2002; Egan et al., 2013; Goecke et al., 2010; Hadfield, 2011; Joint et al., 2000; Mos et al., 2011; Patel et al., 2003; Wang et al., 2012), through the production of bioactive molecules and quorum sensing, whereby microorganisms communicate and coordinate their behavior through the accumulation of chemical cues (Dobretsov et al., 2007; Swift et al., 2001; Waters and Bassler, 2005). For example, enhanced settlement of spores of green algae of the genus *Ulva* on biofilm surfaces was linked to a cell-to-cell communication between prokaryotes and eukaryotes (Joint et al., 2002). Likewise, various strains of *Vibrio* bacteria could stimulate the settlement of *Ulva linza* (Marshall et al., 2006). Conversely, bacteria of the genus *Pseudoalteromonas* inhibited the settlement of spores of marine algae as well as invertebrate larvae (Bernbom et al., 2011; Holmstrom et al., 2002; Holmstrom and Kjelleberg, 1999).

Nevertheless, there is still very limited knowledge on the role of epilithic bacterial biofilms in regulating the settlement and recruitment of Fucales. Our results would suggest that the settlement of *E. amentacea* was affected by the composition of earlier substratum colonizers, in accordance with previous studies showing that settlement rates of spores of macroalgae, such as *Ulva* sp. and *Polysiphonia* sp., depended on biofilm structure (Holmstrom et al., 2002). In addition to the associated microbial community characterized in this study, other features of biofilms, such as biomass, chlorophyll and carotenoid levels, can influence the settlement of benthic organisms (Nelson et al., 2020). Importantly, in this study, the microalgal component was not analyzed, although diatoms are among the first eukaryotic colonizers of submerged artificial surfaces and one of the most conspicuous organisms in marine biofilms and, hence, likely to be implied in the regulation of macroalgal settlement (Evans, 1981; Patil and Anil, 2005).

Greater settlement of *E. amentacea* on tiles from Capraia Island suggests that the biofilm that developed at this site provided more suitable conditions for zygote attachment and subsequent development compared to the coastal sites. Nevertheless, *E. amentacea* settled also on

tiles from coastal sites, suggesting that they could be potentially recolonized by this macroalga, upon successful supply of propagules. Due to the short zygote dispersal, natural recolonization from distant populations appears unlikely (Clayton, 1990; Johnson and Brawley, 1998; Riquet et al., 2021). However, our study provides preliminary evidence that restoring macroalgae of the genus *Ericaria* at sites from which they have disappeared could be a viable conservation strategy, as long as the re-introduction of fertile individuals produced in the laboratory (De La Fuente et al., 2019; Falace et al., 2018; Gran et al., 2022; Verdura et al., 2018) could provide a source of zygotes. Of course, viability of this strategy at our coastal study sites does not necessarily imply viability at sites characterized by different biotic and abiotic conditions. In addition, attempts of restoring *Ericaria* species may fail, despite its ability to settle on primary rocky substrata. As shown by previous studies (Cebrian et al., 2021; Gianni et al., 2017; Savonitto et al., 2021), high consumption rates by herbivores, in particular by the fish *Sarpa salpa* (Linnaeus, 1758), represent a major hurdle for the re-introduction of these seaweeds. Likewise, successful settlement does not necessarily imply that environmental conditions at coastal sites are suitable for these macroalgae to grow and reproduce efficiently enough to sustain a self-maintaining population (Mancuso et al., 2018).

Finally, the present results should be interpreted with caution, since the EMB used in our settlement trials, being developed on tiles deployed in the shallow subtidal, could be representative of EMB that develop on natural rocky surfaces in the lower bathymetric range of *E. amentacea* (i.e., up to about 1 m depth), but not of higher levels on the shore (i.e., the intertidal). Our experiment should be, therefore, seen as a first test of the effects of different EMB on the settlement of *E. amentacea*.

In conclusion, our study suggests that primary rocky surfaces at sites along the coast of Tuscany, in the proximity of a relatively large city and a commercial port could be suitable for the settlement of *E. amentacea*, although not as much as those at the island site from which macroalgal fronds carrying mature conceptacles were collected. Although not able to provide a definitive proof of the role of EMBs in causing differences in the settlement of *E. amentacea* between pristine and degraded sites, our study brings some evidence that the settlement of this species is controlled by variations in composition of biofilms in these environments. As such, assessing the suitability of primary rocky surfaces for the recruitment of *Ericaria* species should be considered as a key step to take before embarking in restoration practices.

## 5. Credit authorship contribution statement

**Ludovica Pedicini:** Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. **Claudia Vannini:** Resources, Investigation, Writing - Review & Editing. **Fabio Rindi:** Methodology, Writing - Review & Editing. **Chiara Ravaglioli:** Investigation, Writing - Review & Editing. **Iacopo Bertocci:** Investigation, Writing - Review & Editing; Validation. **Fabio Bulleri:** Conceptualization, Methodology, Writing - Review & Editing, Validation, Supervision.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.marenvres.2023.106035>.

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