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## Epiphytic algal flora associated with habitat-forming brown seaweed in a central Mediterranean coastal area (Conero Riviera, Adriatic Sea): diversity and relationship with environmental variables

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### Abstract

The *Cystoseira s.l.* complex includes the most common macrophytes in shallow sublittoral habitats of Mediterranean rocky shores, where they form marine forests providing valuable ecosystem services. These communities are considered to be repositories of great marine biodiversity, but the epiphytic assemblages associated with them are still not fully known. In particular, extremely limited information is available about their microalgal epiphytes. We examined the microepiphytic and macroepiphytic communities associated with the brown alga *Gongolaria barbata* in a coastal area of the central Adriatic shore (Conero Riviera, Italy). We recorded 119 microalgal taxa and 60 macroalgal taxa growing as epiphytes on this species. Diatoms were the dominant microepiphytes, accounting for 83% of the total number of taxa, followed by Dinophyceae (9%), unidentified phytoflagellates (4%) and Cyanobacteria (3%). A slight negative and positive correlation was observed between phytoflagellate abundance and water temperature and DIN (Dissolved Inorganic Nitrogen). Red algae represented the majority of the macroepiphytes recorded, accounting for 60% of the total. Most of the macroalgal epiphytes were ephemeral species with filamentous morphology, and the non-indigenous red alga *Melanothamnus japonicus* was among the most common epiphytes. Our results confirm the role of the *Cystoseira s.l.* species as a major repository of marine biodiversity in the Mediterranean. However, they also suggest that our understanding of the diversity of their associated communities is probably still largely incomplete. New studies combining traditional taxonomy and DNA metabarcoding will be essential to advance our knowledge of them.

**Keywords:** Algal forests; *Cystoseira*; ecosystem engineers; *Gongolaria barbata*; macrophytobenthos; microphytobenthos.

### Introduction

Rocky coastal habitats are typically colonized by numerous sessile organisms, including large canopy-forming seaweeds. These organisms are often constructors of biocenoses and enhance biodiversity thanks to their tridimensionality (Sales & Ballesteros, 2009; Casamajor *et al.*, 2019).

Brown seaweeds of the *Cystoseira sensu lato* complex (genera *Cystoseira* C. Agardh, *Ericaria* Stackhouse and *Gongolaria* Boehmer; Fucales, Phaeophyceae) form forests with a high ecological value in temperate rocky coastal habitats and play an especially important role in the Mediterranean Sea (Verdura *et al.*, 2018; Falace *et al.*, 2018; Fabbrizzi *et al.*, 2020; Medrano *et al.*, 2020). As is the case with most large-sized macroalgae, the *Cystoseira s.l.* species are characterized by a densely branched thal-

lus providing numerous microhabitats that can host many different organisms (e.g., small invertebrates, sciaphilous algae adapted to shaded conditions). Some of these adhere directly to the surface of the macroalga (animal and algal epiphytes) and may represent an important food source for larger animals (García-Fernández & Bárbara, 2016).

Assessments of the epibiota associated with *Cystoseira s.l.* have mainly focused on the macroepiphytic communities (Ballesteros, 1988; Lluch & Garreta, 1989; Morales-Ayala & Viera-Rodríguez, 1989; Arrontes, 1990; Ballesteros, 1990a, 1990b; Otero-Schmitt & Sanjuan, 1992; Otero-Schmitt & Pérez-Cirera, 1996; Beleggratis *et al.*, 1999; Simakova, 2009; Sales *et al.*, 2012; Mačić & Svirčev, 2014; Piazzini *et al.*, 2018; Casamajor *et al.*, 2019; Kozak *et al.*, 2020; Prazukin *et al.*, 2020; Afanasyev & Akatov, 2021). Additional investigations of the epiflora

associated with *Cystoseira* assemblages, including a detailed assessment of the epiphytic microphytobenthos, would be of great relevance in environmental monitoring programs since fast shifts in the epiphytic composition can be caused by a deterioration of the physiological state of the host due to external stressors (Karez *et al.*, 2004; Casamajor *et al.*, 2019).

Among all Mediterranean *Cystoseira s.l.* species, *Gongolaria barbata* (Stackhouse) Kuntze is the one that can reach the largest dimensions (the thalli have a cylindrical/conical shape and can be up to 1.3 m high; Cormaci *et al.*, 2012). For this reason, *G. barbata* is one of the Mediterranean macrophytes with the highest potential to host a very rich associated biodiversity. Despite this, however, there is still a large knowledge gap about its associated biota and only a few studies focusing on the epiflora of *G. barbata* are currently available. One was performed along the Montenegro coast (Mačić & Svirčev, 2014) and one in the Black Sea (Deyanova *et al.*, 2010). They both considered only macroalgal epiphytes, reporting respectively 13 (Mačić & Svirčev, 2014), and 41 species (Deyanova *et al.*, 2010). *G. barbata*, however, has a much wider geographical distribution, spanning the whole Mediterranean (Guiry & Guiry, 2023) and the almost non-existent information available for other regions is limited to occasional mentions (e.g., Sauvageau, 1912). Such limited knowledge is remarkable, considering the great ecological relevance of this species and its habitat-forming nature due to its complex morphology.

In this study we present an overview of the diversity of the epiflora (both macroepiphytic and microepiphytic) associated with *G. barbata* in a central Mediterranean coastal area (Conero Riviera, Adriatic Sea). The results of this study provide a set of new data that will be valuable for comparison with other *Cystoseira s.l.* species, as well as populations of *G. barbata* from other Mediterranean locations.

## Materials and Methods

### Study area

The Conero Riviera is a 15 km-long rocky shore located in the central Adriatic coast of Italy. This area is characterized by a combination of environmental features which are rather different from most coastal areas in the Mediterranean: a broad range of variation in surface seawater temperature (from 7–8°C in January to 26–27°C in August) and salinity (33–36, but occasionally as low as 20); a high sediment load and frequent conditions of high water turbidity; a shoreline consisting of cliffs formed by marl, a highly friable calcareous rock, which results in the presence of coarse gravel and cobbles on large portions of the seafloor; and anthropogenic stress resulting from the presence of the large commercial harbour of Ancona and intensive summer tourism. Further details about the hydrological and geological features of the area are available in Rindi *et al.* (2020).

### Sampling of environmental variables

Surface temperature and salinity were measured with a CTD, Model 30 Handheld Salinity, Conductivity and Temperature System, YSI (Yellow Spring, OH USA). Water samples for nutrient analysis were collected on each sampling date and at each sampling site for the microalgal epiphytes (see details below). Water was collected in polyethylene bottles (50 ml) near the sampled macroalga, rapidly carried to the laboratory, filtered using GF/F Whatman filters (25 mm) and stored in triplicates in 4 ml polyethylene bottles at 22° C until analysis.

### Sampling sites and times/periods

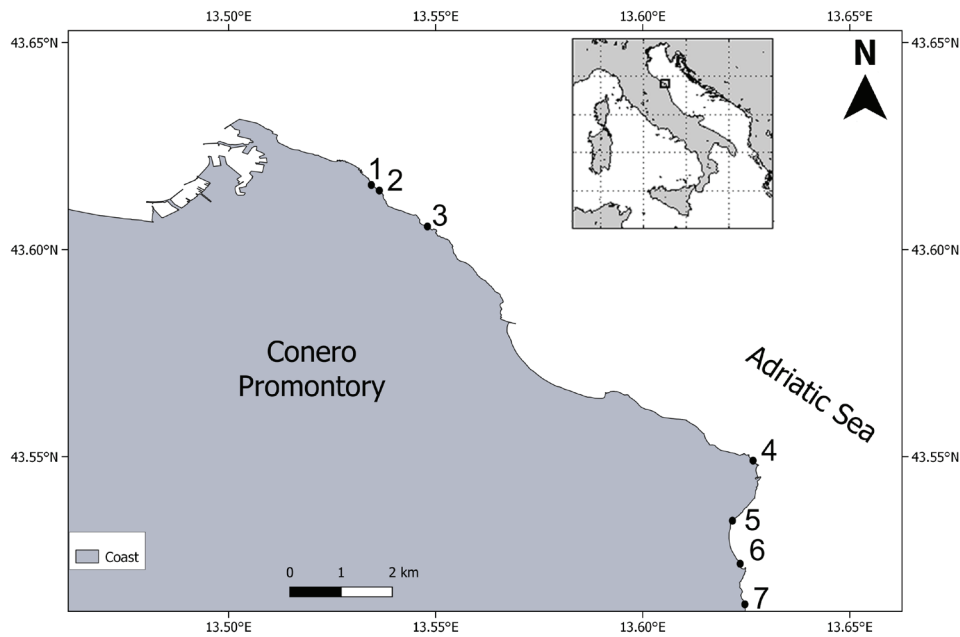
Different strategies were adopted for sampling the microalgal epiphytes and macroalgal epiphytes. Sampling of microepiphytes was carried out in 2017 at 2 sites located in the urban area of Ancona (Piscinetta del Passetto and Scalinata del Passetto, hereafter Piscinetta and Scalinata) (Fig. 1; Table 1). The Piscinetta hosts a fragmented population of *G. barbata* consisting of several large patches growing on a shallow rocky bottom (-1/1.5 m; Fig. S1A). The Scalinata hosts a population consisting of some large, scattered specimens growing on boulders at a depth ranging between -1 and -2 m (Fig. S1B).

Sampling of macroepiphytes was performed during the course of many field surveys of the benthic macroalgal flora carried out in the years 2012–2023 at 7 sites on the Conero Riviera (Fig. 1). Details of these sites are provided in Table 1.

Collections were made by snorkelling. A decision was made to study the microepiphytic and macroepiphytic vegetation growing on fully developed primary branches of *G. barbata* (i.e., branches at least 20 cm long arising from the stipe). A choice was deliberately made to avoid removing the entire thalli of *G. barbata*; given the precarious state of many populations, nowadays this practice should be considered ethically unacceptable for all species of *Cystoseira s.l.* (Rindi *et al.*, 2023).

### Sampling of microepiphytes

Collections were made in a single sampling date from the Piscinetta (22 February 2017) and on four sampling dates in 2017 from the Scalinata (22 February, winter sampling date; 23 March, spring sampling date; 24 July, summer sampling date; and 23 November, autumn sampling date). Three thalli of *G. barbata* were randomly chosen at each site on each date. Two primary branches (i.e., branches issued from the stipe) were collected from each thallus. Each branch was detached at its base and placed in sealable Ziploc bags with the surrounding water, paying attention not to lose any epiphytes. On the same day, the material was transferred to the laboratory, where the samples were placed in plastic jars and immediately treated with an ultrasonic cleaning device (Branson 2220 Ultrasonic Cleaner, Branson, Danbury, CT) to facilitate the detachment of



**Fig. 1:** Map showing the position of the sampling sites for microalgal epiphytes (1 and 2) and macroalgal epiphytes (1, 2, 3, 4, 5, 6 and 7). See Table 1 for details.

**Table 1.** Details of sampling sites.

Sampling site	Approximate GPS coordinates	Sampling	Environmental features
<b>1. Piscinetta del Passetto</b>	43.619167 N, 13.531667 E	Microepiphytes and macroepiphytes	Partially sheltered inlet. Shallow depth (1-1.5 m), horizontal rocky substratum. Urban beach affected by high anthropogenic impact during summer (mainly in the form of trampling). Presence of a fragmented <i>G. barbata</i> population distributed in patches.
<b>2. Scalinata del Passetto</b>	43.618442 N, 13.532006 E	Microepiphytes and macroepiphytes	Wave-exposed. Shallow depth (1-2 m), sub-horizontal rocky substratum. Urban beach frequented by swimmers in summer months. Rocky floor with some large boulders (1-1.5 m wide); <i>G. barbata</i> occurs as scattered specimens growing on the top of such boulders.
<b>3. Spiaggia della Scalaccia</b>	43.605092 N, 13.548812 E	Macroepiphytes	Sheltered to semi-exposed. Shallow (0.5-1.5 m) rocky sub-horizontal substratum. Rock partially covered by coarse gravel and pebbles, hosting a fragmented population of <i>G. barbata</i> in the most sheltered part.
<b>4. Spiaggia delle Due Sorelle</b>	43.547679 N, 13.627653 E	Macroepiphytes	Semi-exposed. Gently sloping bedrock, from 1-1.5 m to 3-4 m depth. Gently sloping rocky substratum partially covered by coarse gravel and cobbles. Popular site for summer tourism, subject to high anthropogenic impact during summer. <i>G. barbata</i> population forming a dense belt at the northern end of the beach.
<b>5. Spiaggia dei Sassi Neri</b>	43.534747 N, 13.622035 E	Macroepiphytes	Wave-exposed. Shallow, gently sloping bedrock (1-1.5 m). Presence of cobbles and some large boulders. Site subject to moderate anthropogenic impact during summer. <i>G. barbata</i> population distributed in dense patches.
<b>6. Spiaggia Urbani</b>	43.518115 N, 13.624357 E	Macroepiphytes	Wave-exposed. Shallow bedrock (1-1.5 m). Gently sloping rocky substratum partially covered by gravel and sand. Moderate-high anthropogenic impact during summer. Southern end of the site hosting large patches of <i>G. barbata</i> .
<b>7. Spiaggia del Frate</b>	43.521008 N, 13.624210 E	Macroepiphytes	Wave-exposed. Shallow (1-1.5 m), gently sloping bedrock. Rocky substratum partially covered by gravel and sand. Moderate anthropogenic impact during summer. Population of <i>G. barbata</i> distributed in large, relatively dense patches.

strongly attached microepiphytes from the algal surface. Sonication cycles lasted for 10 minutes to avoid heating. After ultrasonication, the surface of *G. barbata* was observed under a stereomicroscope to verify the complete removal of epiphytic cells. Additional cycles were required for samples collected in the winter date at the Piscinetta and summer date at the Scalinata. The known volume of water with the detached microalgae was transferred into plastic bottles (Table S1), where the material was fixed with 0.8% (final concentration) prefiltered neutralized formaldehyde and stored in a darkroom at 4°C until analysis.

Then, after gentle homogenization, a 0.5-10 ml subsample was settled into a Utermöhl cylinder/chamber, and observed using an inverted microscope (Axiovert 25, Zeiss), to count epiphytic microalgae. Their identification was based on morphological features (Van Heurck 1880-1885; Hustedt, 1985; Peragallo & Peragallo, 1897-1908; Hasle & Syversten, 1996). During counting, cells were measured to calculate cell biovolumes, which allowed for an estimation of the biomass following Menden-Deuer & Lessard (2000). Abundance and biomass values were expressed as cells g dw<sup>-1</sup> and µg C g dw<sup>-1</sup> of macroalga, respectively.

The microepiphytic diatoms were classified into growth forms: erect and adnate forms, *i.e.* araphid and monoraphid diatoms living attached to the substrate through mucus pads or peduncles at one valve pole and through the entire valve surface, respectively; motile forms, *i.e.* pennate biraphid diatoms able to move over the substratum; tube-dwelling forms, *i.e.* motile species living in mucilage tubes of their own making; and plocon forms, *i.e.* diatom cells loosely associated with substrate surface (Romagnoli *et al.*, 2007; Majewska *et al.*, 2013). The relative abundance of the growth forms was assessed for each sample in terms of number of taxa, total abundance and total biomass. The microepiphytic community was analyzed in its total species composition and richness ( $\gamma$ -diversity). The differences in terms of species number ( $\alpha$ -diversity) between sites were estimated for the data collected on 22 February 2017, the  $\beta$ -diversity was calculated following the Jaccard dissimilarity index ( $b_j$ ):

$$b_j = \frac{b + c}{a + b + c}$$

in which:

$a$  = n° of species in common between Piscinetta and Scalinata

$a_1$  = n° of taxa present in the Piscinetta site

$a_2$  = n° of taxa present in the Scalinata site

$b = a_1 - a$

$c = a_2 - a$

### Sampling of macroepiphytes

Samples of macroepiphytic vegetation were obtained from a branch of the same thalli sampled for the microepiphytes in 2017. For the other collections made during the

period 2012-2023, whole primary branches, or fragments of primary branches, at least 20 cm long, were collected. The samples were examined in the laboratory using a stereomicroscope and a light microscope, on the collection day or shortly afterwards; observations were made on fresh material without use of chemical fixatives. The macroalgal epiphytes were identified at the best possible level of taxonomic resolution based on morphological features (Coppejans 1983; Cormaci *et al.*, 2012; Rodriguez-Prieto *et al.*, 2013; Cormaci *et al.*, 2014, 2017, 2020, 2021, 2023).

The macroepiphytic community was analysed in terms of total species composition and richness ( $\gamma$ -diversity). The species recorded were subdivided in functional groups following Steneck & Dethier (1994) (adding a group defined as tubular for *Ulva cf. intestinalis* Linnaeus, which was not classifiable in any of the groups proposed by these authors).

Voucher specimens of epiphytic macroalgae were deposited in the phycological section of the Herbarium Anconitanum (ANC ALG), Università Politecnica delle Marche, Ancona, Italy.

### Nutrient analysis

The analyses of N-NO<sub>3</sub>, N-NO<sub>2</sub>, N-NH<sub>4</sub>, and P-PO<sub>4</sub> and Si-Si(OH)<sub>4</sub> were performed following the colorimetric method by Strickland & Parsons (1972), using an Autoanalyzer QuAatro Axflow. Detection limits were 0.02 µM for N-NO<sub>3</sub>, N-NO<sub>2</sub>, N-NH<sub>4</sub> and Si-Si(OH)<sub>4</sub> and 0.03 µM for P-PO<sub>4</sub>.

### Statistical analysis

Data of abundance of microepiphytes were analyzed using Statistica (StatSoft Inc., Tulsa, OK, USA) software. The Shapiro-Wilks test was used to check data for normal distribution, while the Levene's test was used to assess homogeneity of variance. Rank-transformed variables were used when tests showed heterogeneous variances and/or non-normal distributions.

Hypotheses concerning differences in (i) abundance and biomass of epiphytic microalgae in each site (only in winter, n=12) and in each sampling date (only at Scalinata, n=24), (ii) abundance of each major group of microphytobenthos (diatoms, dinoflagellates, phytoflagellates and cyanobacteria) in each sampling date (only at Scalinata, n=24) and (iii) abundance of diatom growth forms (adnate, erect, motile, tube-dwelling, plocon and planktonic) in each sampling date (only at Scalinata, n=24) were tested using one-way analyses of variance (ANOVAs). When significant differences for the main effect were detected ( $p < 0.05$ ), a post-hoc Tukey's pairwise test was also performed.

Moreover, all environmental variables were tested for significant correlations (Pearson correlation coefficients) with abundances of each microphytobenthos group.

## Results

### Environmental variables

The temporal variations of temperature, salinity and nutrient concentrations are shown in Figure 2. Temperature ranged from 8.3 to 25.9 °C at the Scalinata, and from 8 to 25.4 °C at the Piscinetta (Fig. 2A). Salinity ranged from 33.7 (winter sampling) to 36.4 (summer sampling) and from 34.0 (winter sampling) to 35.8 (summer sampling) at the Scalinata and Piscinetta, respectively (Fig. 2B).

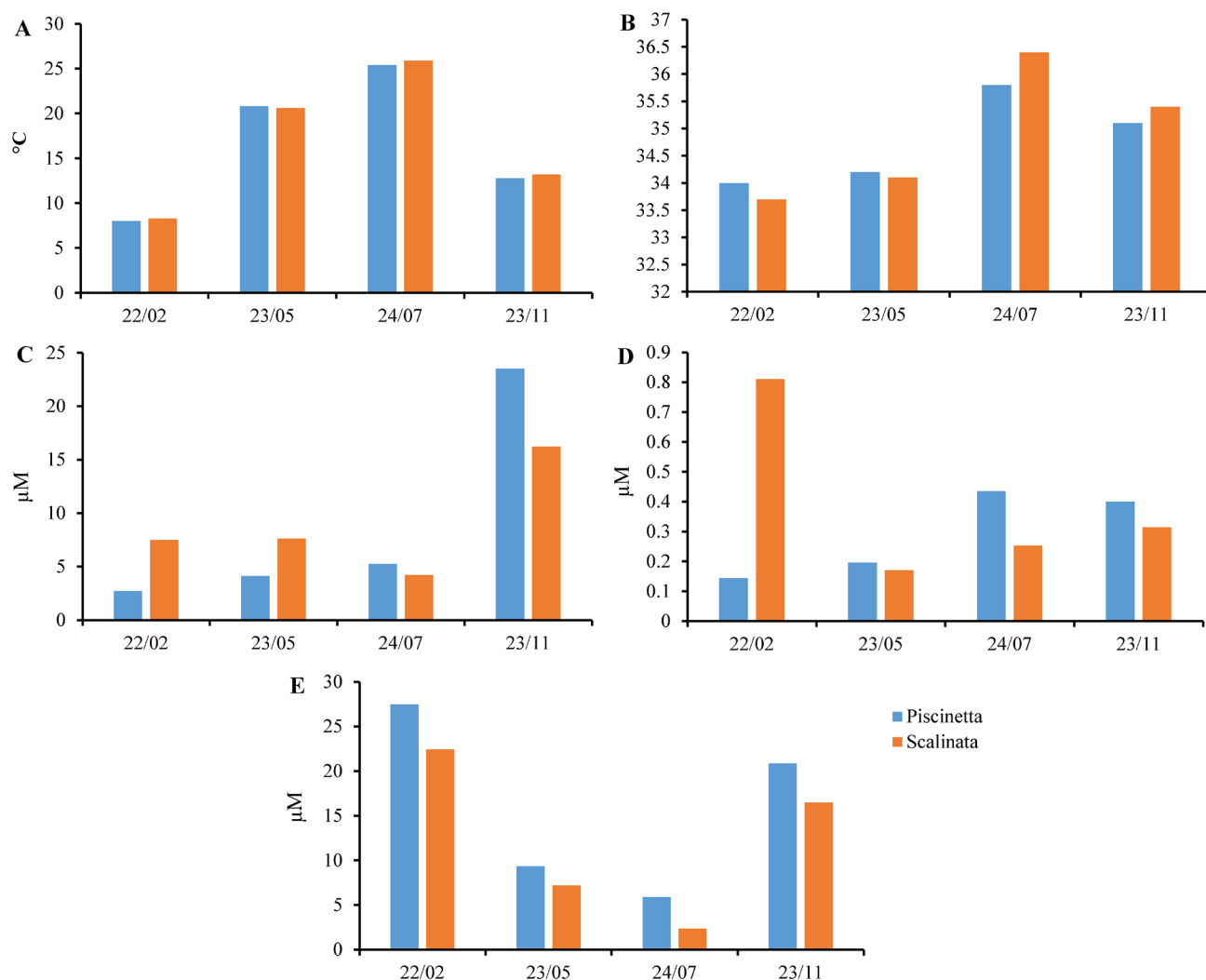
Nutrients showed different patterns at the two studied sites. At the Scalinata, Si-Si(OH)<sub>4</sub> concentration was similar in the winter and spring dates (7.503 and 7.627 μM, respectively) and decreased to 4.214 μM in the summer date, reaching the peak in the autumn date (16.231 μM) (Fig. 2C). At the Piscinetta, an increasing trend of Si-Si(OH)<sub>4</sub> was observed from the winter to the autumn dates, ranging from 2.714 to 23.529 μM, respectively (Fig. 2C).

The P-PO<sub>4</sub> concentration at the Scalinata was 0.811 μmol l<sup>-1</sup> in the winter date, decreased to 0.171 μmol l<sup>-1</sup> in

the spring one, and then increased throughout the summer and autumn dates (0.253 and 0.315 μM, respectively) (Fig. 2D). At the Piscinetta, the lowest P-PO<sub>4</sub> concentration was recorded in the winter date (0.144 μM). The P-PO<sub>4</sub> concentration increased in the spring, reached its maximum value in the summer (0.435 μM), and then decreased in the autumn (0.401 μM) dates (Fig. 2D).

Overall, a higher concentration of Dissolved Inorganic Nitrogen (DIN), obtained as the sum of nitrate (N-NO<sub>3</sub>), nitrite (N-NO<sub>2</sub>) and ammonium (N-NH<sub>4</sub>), was measured at the Piscinetta than at the Scalinata in each sampling date. In both sites, DIN showed a decreasing trend from winter to summer dates, when the lowest concentrations were recorded, then increasing again in the autumn date (Fig. 2E). At the Scalinata, the lowest DIN concentration was 2.378 μM in the summer date, while the highest value was recorded in the winter date (22.432 μM). At the Piscinetta, DIN concentrations ranged from 5.893 μM in the summer date to 27.466 μM in the winter date.

Temperature values were significantly and negatively correlated with DIN ( $r = -0.9741$ ,  $n = 8$ ,  $p < 0.001$ ). No correlation was detected with other environmental parameters.



**Fig. 2:** Patterns of the measured environmental variables at the Piscinetta and Scalinata study sites on the four sampling dates (in 2017) for the microepiphytes. A): temperature; B): salinity; C): Si-Si(OH)<sub>4</sub>; D): P-PO<sub>4</sub>; E): Dissolved Inorganic Nitrogen.

### Microepiphytic community and relationships with environmental variables

In the course of the study, 119 microalgal taxa were found as epiphytes of *G. barbata*, among which 47 could be identified at specific level (Table S2; Fig. 3). For the others, observation in light microscopy was not sufficient to observe diagnostic characters at species level. For some taxa, the extremely small size or simple morphology did not allow for a species-level discrimination, so they were lumped into collective categories (e.g., unidentified cryptophytes, unidentified phytoflagellates). Diatoms were the dominant group, accounting for 83% of the total number of taxa, followed by dinoflagellates, unidentified phytoflagellates and cyanobacteria (Table S3). Within the diatoms, pennate forms were dominant over centrics (Table S2).

The highest number of microalgal taxa was recorded at the Scalinata in the autumn date (60 taxa), whereas the lowest number was found at the same site in the winter date (47 taxa). Twenty-one taxa were consistently present in every sample collected at the Scalinata, and among them 18 were also found in the Piscinetta. In the winter date, the Piscinetta hosted a higher taxon richness than the Scalinata (51 and 47 taxa, respectively), with  $b_1 = 58\%$  (Table S4).

In terms of growth forms, motile diatoms were the most represented group (55%), followed by adnate

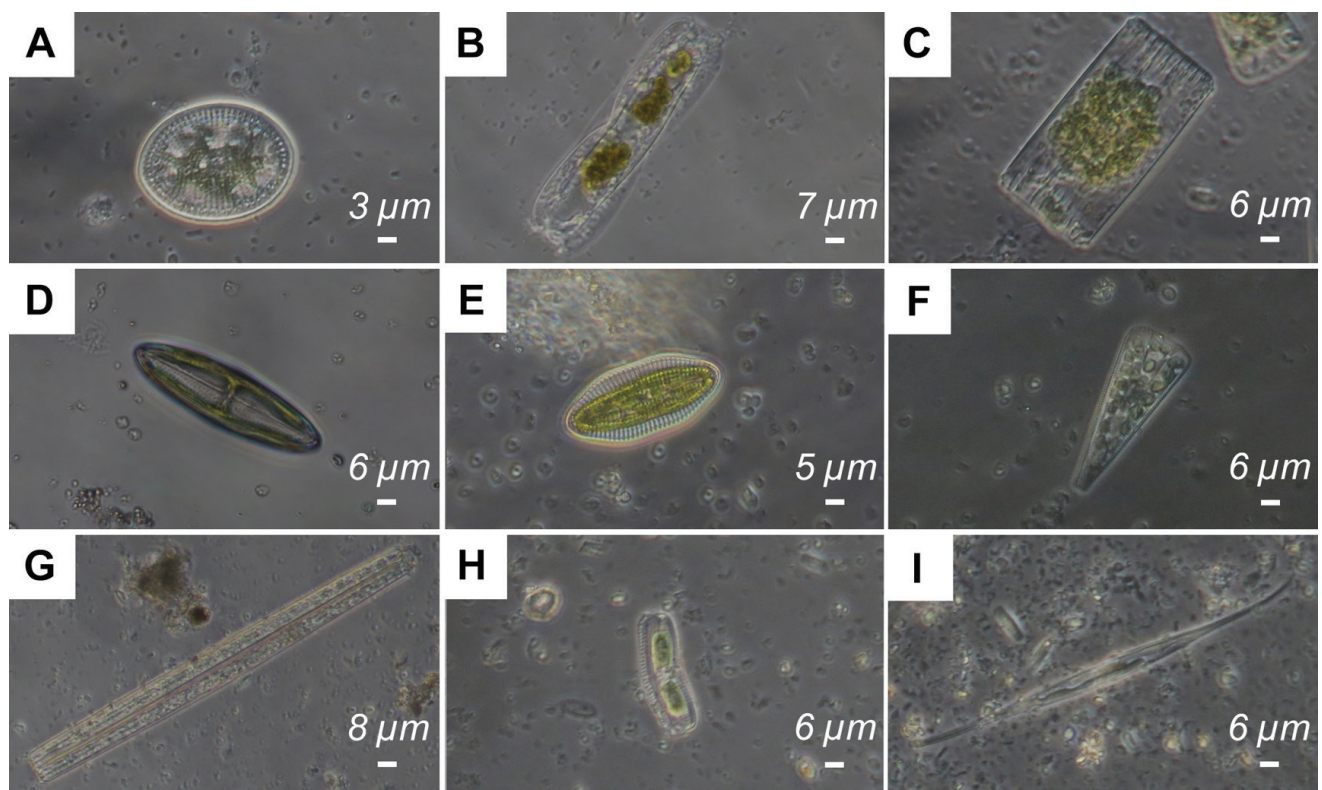
forms (18%) and erect forms (17%), plocon (8%), and tube-dwelling forms (2%). Even considering each sampling date individually, the motile forms remained the most represented.

In the winter sampling, the abundance of epiphytic microalgae on *G. barbata* showed significantly higher values at the Piscinetta than at Scalinata (Table S5,  $p < 0.001$ ). Significantly higher biomass values were recorded at the Piscinetta than at the Scalinata (Table S6,  $p < 0.001$ ).

For the Scalinata, differences in microepiphyte abundances were observed among sampling dates. Significantly higher abundances were observed in the summer sampling than in the others ( $p < 0.001$ ), while no significant differences were observed among the winter, spring, and autumn samplings (Table S7). In the same way, higher biomass values were observed in the summer sampling than in the winter, spring, and autumn ones, although significant differences were detected only between summer and autumn (Table S8).

Significantly higher abundances of diatoms and cyanobacteria were observed in the summer sampling than in the winter, spring, and autumn ones ( $p < 0.01$ , 0.01 and 0.001, respectively), while no significant differences among sampling dates were observed in the abundances of dinoflagellates and phytoflagellates (Table S9).

In terms of growth forms, motile diatoms showed significantly higher abundances than all other forms (Table S10,  $p < 0.01$ ), except for erect diatoms. Moreover, erect,



**Fig. 3:** Habit of some common microalgal epiphytes of *Gongolaria barbata* recorded in the study. (A) *Cocconeis scutellum* valve view (adnate growth form). (B) *Tropidoneis lepidoptera* girdle view (motile growth form). (C) *Striatella unipunctata* girdle view (erect growth form). (D) *Trachineis aspera* valve view (motile growth form). (E) *Diploneis cabro* var. *subelliptica* (motile growth form). (F) *Licmophora abbreviata* girdle view (erect growth form). (G) *Synedra* sp., two cells attached along the valve view that are showing their girdle (erect growth form). (H) *Achnanthes brevipes* girdle view (erect growth form). (I) *Gyrosigma fasciola* valve view (motile growth form).

and motile diatoms showed higher abundances in the summer date than in the others (Table S11,  $p < 0.001$ ) at the Scalinata.

Pearson coefficients did not reveal any correlation between composition and abundance of the whole microphytobenthic community and temperature, salinity, and nutrient concentrations.

A slight negative and positive correlation was observed only between phytoflagellates abundances and water temperature values ( $r = -0.790$ ,  $p < 0.05$ ) and DIN ( $r = 0.779$ ,  $p < 0.05$ ), respectively.

### Macroepiphytic community

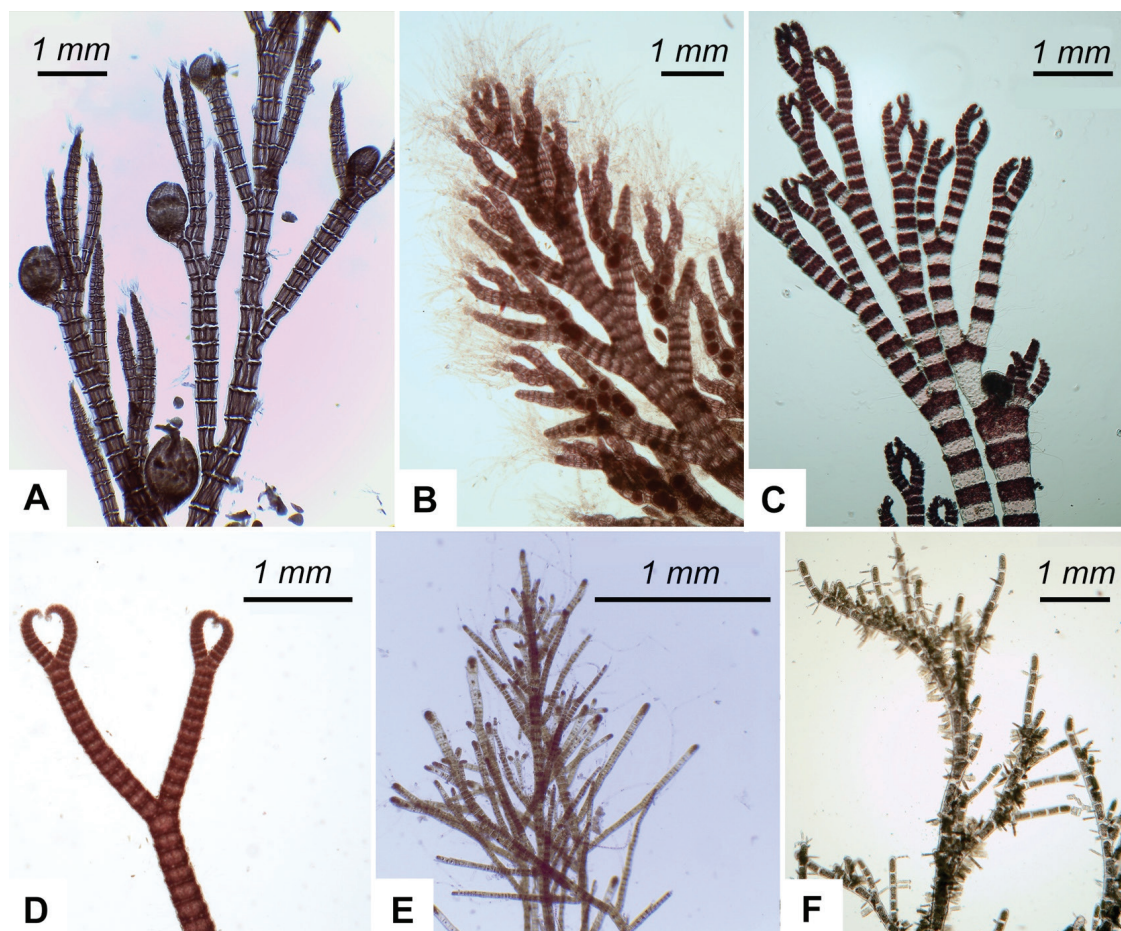
In terms of sampling effort, the Piscinetta was the site for which most frequent sampling was possible (36 sampling dates; Table S12). Due to limited accessibility, the least sampled sites were Spiaggia dei Sassi Neri and Spiaggia Urbani, for which only 1 sampling was possible.

Sixty macroalgal taxa were recorded as epiphytes of *G. barbata*, 45 of which could be identified at species level (Table S13; Fig. 4). For the remaining macroalgae, a species-level identification was not possible due to the small size of the specimens or inability to observe critical diagnostic characters; it is likely, however, that in several cases these were juvenile forms of other taxa that were

identified at species level. Red algae (Rhodophyta) were the most diverse group (Table S14). Substantial differences in  $\alpha$ -diversity were found among the 7 sites. The site with the highest  $\alpha$ -diversity was the Scalinata (37 macroalgae, of which 26 identified at the species level), while the lowest was found at Spiaggia dei Sassi Neri (1 species). Such differences clearly reflected differences in sampling effort (due to easy access, Piscinetta and Scalinata were sampled much more often than the other sites).

*Vertebrata fruticulosa* (Wulfen) Kuntze, *Melanothamnus japonicus* (Harvey) Díaz-Tapia & Maggs and *Sphacelaria cirrosa* (Roth) C. Agardh were the species most frequently recorded (Table S13). The most widely distributed (present in more than half of the monitored sites) were *Vertebrata fruticulosa* (6 sites), *Sphacelaria cf. cirrosa* (4 sites), *Ceramium virgatum* Roth (4 sites), *Nitophyllum punctatum* (Stackhouse) Greville (4 sites), *Osmundea truncata* (Kützinger) K. W. Nam & Maggs (4 sites) and *Dicotyota dichotoma* (Hudson) J. V. Lamoroux (4 sites).

Filamentous algae were the most represented and diverse functional group (34 taxa; Fig. S2A, S2B). Within them, the main contributors were red algae (48%), followed by green and brown algae (Table S15). Conversely, the least represented functional groups were tubular and crustose algae, which were represented respectively by the green alga *Ulva cf. intestinalis* and a few encrusting corallines.



**Fig. 4:** Habit of some common macroalgal epiphytes of *Gongolaria barbata* recorded in the study. (A) *Melanothamnus japonicus*. (B) *Vertebrata fruticulosa*. (C) *Ceramium siliquosum*. (D) *Ceramium virgatum*. (E) *Sphacelaria cirrosa*. (F) *Cladophora* sp., densely covered by epiphytic diatoms.



## Discussion

### *Diversity of the micro- and macroalgal epiphytic communities*

Our results represent a major advancement in the knowledge of the biodiversity associated with *Cystoseira s.l.* in the Mediterranean, namely as regards the microepiphytic component. This is the first study to present data on the microalgal vegetation associated with *Gongolaria barbata*. In general, data on the microalgal communities associated with Mediterranean *Cystoseira s.l.* are extremely scarce and currently limited to a single species, *Cystoseira compressa* (Esper) Gerloff & Nizamuddin. Such information was published in two recent studies (Lenzo *et al.*, 2022, 2023) based on collections made at one of the two sites that we sampled in this study (Piscinetta del Passetto). This lack of knowledge is surprising, given the importance of microphytobenthos in trophic webs and its involvement in facilitation cascades, and considering that *Cystoseira s.l.* species, with their large size and branched habit, presumably host very diverse microalgal communities.

Our study revealed a high diversity of taxa for both microalgal and macroalgal epiphytes. For the macroalgae, the number of taxa recorded is higher than those reported in previous studies on *G. barbata* in other Mediterranean regions: 60 taxa compared to 41 taxa recorded by Deyanova *et al.* (2010), and 31 recorded by Mačić & Svirčev (2014). These results are remarkable considering that, due to limitations relating to sampling strategies, the recorded taxa certainly do not cover the whole diversity of the studied communities. Microscopic examination of the microepiphytes was demanding in terms of workload; this did not allow to extend the sampling to more than two sites and four sampling dates. For the macroepiphytes, the spatial coverage was very uneven in terms of sampling effort, since two sites were sampled much more often than the others. In addition, our data were obtained only from a part of the thallus, the primary branches, which is non-perennial. We took care not to remove whole thalli of *G. barbata* to avoid depleting the sampled populations; this was a critical aspect to consider because in the study area species of *Cystoseira s.l.* appear to have undergone a long-term regression, which can be attributed to a negative combination of high nutrients, sediment load and grazing pressure (Irving *et al.*, 2009; Ferrario *et al.*, 2015; Strain *et al.*, 2015; Rindi *et al.*, 2020). This, however, prevented an assessment of the epiphytes growing on the basal disk and the stipe, which are the perennial parts of the thallus. As such, they are permanently exposed to the colonization of epiphytes and can be expected to support a higher epiphytic load (e.g., Fig. S2C). Indeed, it is known that differences in the longevity (time available for epiphytes to develop) of different portions of a basiphyte can influence the distribution of the epiphytes (Lavery & Vanderklift, 2002). In the case of branched seaweeds such as *Cystoseira s.l.*, differences in longevity also involve differences in thallus microhabitat features, such as extent of shelter from high light irradiance (photophilous epiphytes growing in the upper parts

of the thallus, sciaphilous species in the basal parts), as well as shelter from hydrodynamism and grazing provided by the branches (Lluch & Garreta, 1989; Morales-Ayala & Viera-Rodríguez, 1989; Arrontes, 1990; Otero-Schmitt & Pérez-Cirera, 1996). Differences in surface roughness and defensive capabilities of different parts of the thallus (i.e., antigrazing compounds distribution) may also contribute to these differences (Arrontes, 1990). Arrontes (1990) provided a description of the distribution pattern of the epiphytes on the host based on their morphology and life cycle, indicating ephemeral filamentous epiphytes as the most abundant on apical parts of *Gongolaria baccata* (S. G. Gmelin) Molinari & Guiry. Conversely, larger and long-living corticated epiphytes were concentrated in the lower part of the thallus. Future studies might reveal similar patterns in *G. barbata*.

### *Microepiphytic community*

In general, there is a major knowledge gap concerning microepiphytes growing on seaweeds (Welker *et al.*, 2002; Totti, 2003; Totti *et al.*, 2007; Accoroni *et al.*, 2016). The studies of Lenzo *et al.* (2022, 2023) allow for a comparison of the microepiphytic communities we found on *G. barbata* at the Piscinetta with those recorded on *C. compressa* at the same site in more recent years (2017 in our study, 2018 and 2021 in the studies of Lenzo *et al.*). This revealed that 16 diatoms and 3 dinoflagellates (Lenzo *et al.*, 2022), and 10 diatoms and 10 dinoflagellates (Lenzo *et al.*, 2023) were present both on *G. barbata* and on *C. compressa* respectively in 2018 and 2021 (Table S16). In general, 10 diatoms and 2 dinoflagellates were common to the three studies. Such similarities suggest that these microalgal taxa are common at this site and probably generalist in terms of host association, being able to settle on the surface of several macroalgal species. The local microepiphytic community might have a core composition which is consistent in time; however, it is important to consider the morphological similarities between *G. barbata* and *C. compressa* as well, which likely contribute to such similarity.

The number of identified taxa and composition of the microepiphytic community unraveled in this study are comparable to those of other studies that examined epilithic and epiphytic microalgae at different sites in the same region (Totti, 2003; Totti *et al.*, 2007; Accoroni *et al.*, 2016). Accoroni *et al.* (2016) compared the species richness of microalgal communities growing on pebbles and macroalgae and found a lower diversity in the epiphytic community compared to the epilithic one. It is important, however, to note that the analysed macroalgae were *Ulva* sp. and *Dictyota dichotoma* (Hudson) J.V. Lamouroux. These seaweeds are characterized by a foliose thallus that represents an unstable substratum and is known to host a lower diversity than highly branched thalli (such as the ones of *Cystoseira s.l.* species) (Thomas & Jiang, 1986).

In terms of taxa recorded, diatoms were the dominant group, in agreement with previous studies carried out

in the same geographic area (Accoroni *et al.*, 2016), as well as in other regions (Al-Harbi & Abu Affan, 2016). In general, it is well known that diatoms are the main component of microphytobenthos in temperate regions (Totti, 2003; Totti *et al.*, 2007, 2009; Accoroni *et al.*, 2016). Within this group, the dominance of pennate over centric forms was evident, which is also consistent with other studies (Totti *et al.*, 2007; Accoroni *et al.*, 2016; Al-Harbi & Abu Affan, 2016). The abundance of different microphytobenthic groups seems to be influenced by the nutrient budget, as the N:P ratio strongly affects the composition of the microalgal community (Totti, 2003). Accoroni *et al.* (2016) found the abundances of benthic diatoms positively correlated with total inorganic nitrogen, in agreement with results for the Venice lagoon (Facca *et al.*, 2002). For the only sampling in which both sites were sampled, higher diversity and abundances of microphytobenthos was recorded at the Piscinetta than at the Scalinata. This could be explained considering that at the Piscinetta DIN levels were generally higher than at the Scalinata. In addition, microphytobenthos distribution is also known to be related to spatial patterns of hydrodynamism (Totti, 2003). The Piscinetta is more sheltered than the Scalinata and may provide a more favorable environment for the settlement and survival of benthic microalgae with different adhesion capabilities (Tanaka, 1986).

In this study, maximum abundance and biomass values of microphytobenthos were observed in summer, while minimum in autumn, in agreement with reports for the same (Accoroni *et al.*, 2016) and other areas (Cibic *et al.*, 2007; Méléder *et al.*, 2007; Cibic *et al.*, 2009). The abundance and composition of benthic microalgae is affected by several environmental variables such as light irradiance, hydrodynamic regime, salinity, and nutrients (Coleman & Burkholder, 1994; Snoeijs, 1994; Wear *et al.*, 1999; Hillebrand & Sommer, 2000; Marks & Power, 2001; Frankovich *et al.*, 2006; Snoeijs, 2016), biological mechanisms such as grazing (Nelson, 1997; Hillebrand *et al.*, 2000; Worm & Sommer, 2000) and adhesive strength of diatoms (Tanaka, 1986). The motile growth forms represented the main fraction of the total diversity and abundances in our study, in agreement with the results of Accoroni *et al.* (2016). Indeed, the capability of motile benthic microalgae to move within the mature biofilm confers them a competitive advantage over the other forms (DeNicola & McIntire, 1990). Moreover, although erect microalgae can get better exposure to light and nutrients, they can also be subject to more intense grazing pressure (Tuji, 2000; Wellnitz & Ward, 2000). Accordingly, Totti *et al.* (2007) and Accoroni *et al.* (2016) observed a decrease of erect forms coupled with an increase of adnate forms during summer, when the grazing pressure is higher. However, this was not observed in our study as erect diatoms (as well as motile diatoms and diatoms in general) were significantly more abundant in summer than in the rest of the year, probably due to the well-known high resistance to grazing of *Cystoseira s.l.* (Mannino & Micheli, 2020). This could also explain the higher total abundances of microphytobenthos in this season compared to the others.

### **Macroepiphytic community**

In general, the macroalgal epiphytic vegetation of *G. barbata* on the Conero Riviera is composed of species that are common in the Mediterranean and have been reported as macroepiphytes of other *Cystoseira s.l.* species (Belegratis *et al.*, 1999; Deyanova *et al.*, 2010; Sales *et al.*, 2012; Mačić & Svirčev, 2014; Piazzzi *et al.*, 2018; Afanasyev & Akatov, 2021). Some filamentous macroalgae appear to be recurrent epiphytes of *Cystoseira s.l.* in the Mediterranean, for example the red alga *Vertebrata fruticulosa* and the brown alga *Sphacelaria cirrosa* (Gómez Garreta *et al.*, 1982; Ballesteros 1988, 1990b; Belegratis *et al.*, 1999; Sales *et al.*, 2012; Piazzzi *et al.*, 2018), which were frequently recorded in our samples. *V. fruticulosa* was the most common macroepiphyte of *G. barbata* on the Conero Riviera, having been found in 6 of the 7 study sites. In this area the species occurs mostly as epiphyte on *G. barbata* and *C. compressa*, unlike other Mediterranean regions where it is mainly epilithic (Rindi & Cinelli, 2000). Conversely, the complete absence of articulated corallines of the genus *Jania* (namely, *Jania rubens* (Linnaeus) J. V. Lamouroux and *Jania virgata* (Zanardini) Montagne) is a striking feature of the epiphytic macroflora of *G. barbata* in this area, because these calcified seaweeds are among the most common epiphytes on *Cystoseira s.l.* (Ballesteros, 1988, 1990b; Serio, 1994; Sales & Ballesteros, 2010; Sales *et al.*, 2012; Mačić & Svirčev, 2014; Piazzzi *et al.*, 2018). For the Conero Riviera these species were reported only in early historical studies (1941-1976) and no recent records are available. Rindi *et al.* (2020) suggested that the disappearance of these algae is part of a long-term change that has characterized the macroalgal flora of this area since the 40s of last century.

The works of Deyanova *et al.* (2010) and Mačić & Svirčev (2014) allow a comparison of the macroepiphytic vegetation of *G. barbata* of the Conero Riviera with those of other geographical areas (Black Sea and Montenegro). Based on these studies, 3 species (*Callithamnion corymbosum*, *Sphacelaria cirrosa* and *Chaetomorpha aerea*) and 6 genera (*Callithamnion*, *Ceramium*, *Chaetomorpha*, *Cladophora*, *Sphacelaria* and *Ulva*) appear to be common to these three areas. Comparing our results with these studies, 7 species and 6 more genera reported by Deyanova *et al.* (2010) were also present in our samples; and 5 species and 4 more genera reported by Mačić & Svirčev (2014) also occurred in our samples (Table S17). Such comparison suggests that the epiphytic community of *G. barbata* is characterized by substantial biogeographical variability on large spatial scales, in agreement with similar conclusions obtained for other *Cystoseira s.l.* species by Sales *et al.* (2012) and Piazzzi *et al.* (2018). Further investigations of the epiphytic community of *G. barbata* in other Mediterranean regions are necessary to gain further insights into these patterns.

## Conclusions and directions for future studies

Overall, the results of this study support the generally accepted notion that forests of habitat forming *Cystoseira s.l.* in the Mediterranean represent a major repository of biodiversity. However, they also suggest that our understanding of this diversity is still widely incomplete. This is especially true for the microalgal component, for which accurate sampling and taxonomic identification are problematic. Many of the microalgal taxa observed in this study could not be identified at species, or, sometimes, not even genus level, due to the insufficient resolution of optical microscopy. We recommend that future studies should be based on an integrative taxonomic approach, combining both observations with higher resolution (Scanning Electron Microscopy) and molecular methods (DNA barcoding and metabarcoding), which have the potential to unravel a great deal of diversity that cannot be assessed by traditional optical microscopy.

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## Supplementary Material

The following supplementary material is available for this article:

**Fig. S1:** (A) Detail of the population of *Gongolaria barbata* of the Piscinetta del Passetto. (B) Detail of a thallus of the *Gongolaria barbata* population of the Scalinata del Passetto.

**Fig. S2:** Habit of thalli of *Gongolaria barbata* of the Conero Riviera densely covered by macroalgal epiphytes. (A) Thalli of the Piscinetta del Passetto (18 March 2023), covered by filamentous epiphytes (mainly *Vertebrata* spp., *Melanothamnus japonicus* and *Sphacelaria* cf. *cirrosa*). (B) Detail of population of Spiaggia delle Due Sorelle (03 September 2021); the branches of *Gongolaria barbata* are epiphytized mainly by *Vertebrata fruticulosa*. (C) Detail of the stipe of a thallus of the population of Spiaggia delle Due Sorelle densely epiphytized by *Ulva* cf. *lacinulata* (03 September 2021).

**Table S1.** Water volume in each replicate for the sampling of microalgal epiphytes of *Gongolaria barbata*.

**Table S2.** Microalgal epiphytes recorded on *Gongolaria barbata*. Algal nomenclature and classification follow AlgaeBase (Guiry & Guiry, 2023).

**Table S3.** Percent contribution of microalgal groups to the total taxonomic diversity.

**Table S4.** Comparison between Piscinetta del Passetto and Scalinata del Passetto for the winter date (22 February 2017) in terms of alpha diversity and beta diversity of microalgal epiphytes (Jaccard Index).

**Table S5.** Abundances of epiphytic microalgae at the Piscinetta and Scalinata sites and results of the One-Way ANOVA testing for differences among sampling sites. Mean abundances (cells g dw<sup>-1</sup>) ± standard error (SE). ns, not significant; \*, p < 0.05; \*\*, p < 0.01; \*\*\*, p < 0.001.

**Table S6.** Biomasses of epiphytic microalgae at the Piscinetta and Scalinata sites and results of the One-Way ANOVA testing for differences among sampling sites. Mean abundances (µg C g dw<sup>-1</sup>) ± standard error (SE). ns, not significant; \*, p < 0.05; \*\*, p < 0.01; \*\*\*, p < 0.001.

**Table S7.** Abundances of epiphytic microalgae at the Scalinata site and results of the One-Way ANOVA testing for differences among sampling dates. Mean abundances (cells g dw<sup>-1</sup>) ± standard error (SE). ns, not significant; \*, p < 0.05; \*\*, p < 0.01; \*\*\*, p < 0.001.

**Table S8.** Biomasses of epiphytic microalgae at the Scalinata site and results of the One-Way ANOVA testing for differences among sampling dates. Mean biomass (µg C g dw<sup>-1</sup>) ± standard error (SE). ns, not significant; \*, p < 0.05; \*\*, p < 0.01; \*\*\*, p < 0.001.

**Table S9.** Abundances of diatoms, dinoflagellates, phytoflagellates and cyanobacteria at Scalinata site and results of the One-Way ANOVAs testing for differences among sampling dates. Mean abundances (cells g dw<sup>-1</sup>) ± standard error (SE). ns, not significant; \*, p < 0.05; \*\*, p < 0.01; \*\*\*, p < 0.001.

**Table S10.** Abundances of diatom growth forms at Scalinata site and results of the One-Way ANOVAs testing for differences among growth forms. Mean abundances (cells g dw<sup>-1</sup>) ± standard error (SE). ns, not significant; \*, p < 0.05; \*\*, p < 0.01; \*\*\*, p < 0.001.

**Table S11.** Abundances of diatom growth forms at Scalinata site and results of the One-Way ANOVAs testing for differences among sampling dates. Mean abundances (cells g dw<sup>-1</sup>) ± standard error (SE). ns, not significant; \*, p < 0.05; \*\*, p < 0.01; \*\*\*, p < 0.001.

**Table S12.** Detail of sampling dates of macroalgal epiphytes for each sampling site.

**Table S13.** Macroalgal epiphytes recorded on *Gongolaria barbata*. Algal nomenclature and classification follow AlgaeBase (Guiry & Guiry, 2023).

**Table S14.** Number of species/taxa and percentage contribution of taxonomic macroalgal groups to the total taxonomic diversity.

**Table S15.** Number of species/taxa of macroalgal functional groups and percentage contribution of taxonomic macroalgal groups to each functional category.

**Table S16.** Microalgal diversity at the Piscinetta site: comparison of the results of the present study with those of Lenzo *et al.* (2022, 2023).

**Table S17.** Comparison of the epiphytic macroalgal vegetation recorded in the present study with the results of Deyanova *et al.* (2010) and Mačić & Svirčev (2014); “spp.” was added when same genera but different species were present in different studies. Only macroalgal taxa common to at least two of the considered studies are included in the list.