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Understanding the Role of Macroalgal Complexity and Allelochemicals Production in Invasive and Non-Invasive Macroalgae in the North-Western Adriatic Sea: Effect on the Associated Communities

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Abstract: Highly diverse microphyto and meiobenthic communities are associated with large-sized marine macroalgae. Both morphological traits and allelochemical responses of macroalgae affect the composition of these communities, but the relative importance of these factors remains incompletely understood. In this study we investigated the microphytobenthic and meiobenthic communities associated with some native macroalgae and a non-indigenous species (*Sargassum muticum*) of the north-western Adriatic Sea. These seaweeds were sampled in two coastal sites subjected to different impacts. The possible effects of the structural complexity of the macroalgae and the potential role of allelochemicals (specifically polyunsaturated aldehydes, PUAs) on the associated communities were examined using univariate and multivariate analyses. The results indicate that distinct assemblages were associated with the macroalgae collected at the two different sites. Differences in microphytobenthic communities could be ascribed to differences in the macroalgal morphological traits and in their PUAs production. Conversely, variation of the meiobenthic community seemed to be related mainly to differences in the macroalgal communities at the two sites. This apparent inconsistency between the two analyzed communities suggests that microphytobenthos and meiofauna were differently shaped by the environmental habitat provided by macroalgae in the two sites, that are subjected to different environmental conditions and human activities. Overall, these results indicate that interactions between organisms belonging to different trophic groups (e.g., microphytobenthos and meiofauna) should be investigated in detail to better understand the global role of macroalgae as habitat formers on coastal ecosystems, especially in the case of large-sized introduced species.

Keywords: macroalgal structural complexity; polyunsaturated aldehydes; microphytobenthos; meiofauna; non-indigenous species; chemical ecology

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1. Introduction

In the marine environment, seaweeds are key primary producers and habitat formers, providing space, shelter, and food for a variety of associated epifaunal organisms [1] and substrate for numerous small-sized algal epiphytes [2]. The Mediterranean Sea is a global hotspot of marine biodiversity and its macroalgal flora consists of approximately 1200 species [3]. Due to its semi-enclosed nature, it is believed that human impacts are proportionally stronger in the Mediterranean than in any other sea of the world [4]. Non-indigenous macroalgae are particularly likely to become invasive because of their high reproductive rates, production of specialized metabolites which could act as allelochemicals, and perennial status that make them more competitive than native species [5]. Globally, the impacts of invasive macroalgal populations are typically expressed as community

dominance through space monopolization and changes in the competitive relationships with native assemblages. Invasive aliens can outcompete native species for space, light, or nutrients, creating monospecific stands and homogenized habitats [6], causing changes in community structure, food web structure, and ecosystem processes [7].

Both invasive and native macroalgae are known to produce a variety of allelopathic substances such as phenolic compounds, alkaloids, peptides, oxoacids, and polyketides, including polyunsaturated fatty acids (PUFAs) [8–10] and their derivatives such as polyunsaturated aldehydes (PUAs) [11–13]. Among these natural products there are molecules well-known to influence the abundance and distribution of marine organisms and to play important roles in their inter and intraspecific interactions.

The brown alga *Sargassum muticum* (Yendo) Fensholt is considered one of the most invasive seaweeds in temperate ecosystems. This macroalga was introduced in Europe in the early 1970s and nowadays it is distributed from Norway to Morocco, as well as in the Mediterranean Sea [14]. Particularly, in Italian coastal areas, such as in the north-western Adriatic Sea (i.e., canals in Venice and area of Ancona) and the Mar Piccolo of Taranto, some port areas have been colonized by *S. muticum*, probably due to the intense and frequent maritime traffic [15]. Specifically, in the port of Ancona, the presence of this species has been known since 2009 [16]. Several studies have shown that, in addition to the presence of *S. muticum*, changes observed in invaded locations included a significant reduction in the abundance of previously dominant species [17,18].

Recently, chemical ecologists have also started to consider how research on natural products might be useful in understanding the dynamics of marine biological invasions, assessing their impact in the invaded areas, and evaluating the effects on other native organisms [19]. Allelopathic interactions between different algal species may have different effects on associated organisms, including loss of motility, cellular deformation [20,21], loss of pigmentation, aggregation of the cytoplasm, formation of vesicles, and cell lysis [22]. In particular, polyunsaturated aldehydes (PUAs) can inhibit photosynthesis and growth rates of other algal species, as well as grazing pressure, and can have a teratogenic effect on associated benthic organisms [23]. This was shown in a recent study [24], in which PUAs produced by two macroalgae (*Cystoseira compressa* and *Dictyopteris polypodioides*) were characterized at different sampling times to evaluate their role on structuring the meiobenthic and microphytobenthic communities and were found to affect some of the grazers rather than the entire community structure. Among PUAs, long-chain compounds (i.e., C14–C16) showed stronger effects on the abundances of some microalgal genera and meiobenthic taxa (e.g., harpacticoid species) than short-chain ones (i.e., C6:2).

Meiofauna represents the most abundant and taxonomically diverse metazoan assemblage on Earth [25] and plays a key role in the exchange of organic matter [26,27] as part of the "small food web" (size class 45–1000 µm). Moreover, it supports most of the higher trophic levels [25], being an important food resource for macrofauna, small fish, and other epibenthic predators [28]. Conversely, microphytobenthos communities consist of microalgae associated with benthic substrata. These assemblages often include settled cells or colonies of phytoplanktonic species [29]. Diatoms are usually the main component of microphytobenthos communities in temperate regions and, like macroalgae, are known to produce different PUA compounds [12]. Thus, seaweed aggregations and microphytobenthic biofilms can interact directly or indirectly, influencing the recruitment of associated organisms. Relationship between macroalgae and epiphytic microalgae is considered as food and habitat provision for other associated assemblages. In our previous work [24], a clear separation of the meiofauna and microphytobenthos assemblages was found for the two studied macroalgae, with different temporal trends, thus confirming a seasonal dynamic; moreover, results indicated that macroalgal complexity was a major determinant of the meiofaunal community structure, rather than PUAs which showed species-specific effects.

Introduced species of marine macroalgae may be morphologically dissimilar from native species. Indeed, structural complexity of macroalgae could influence associated

communities driving their diversity, abundance, and community structure [30]. If the introduced macroalga is a large-sized species, it will produce a novel habitat available for the colonization of local native species. Changes in habitat structure can be propagated to food webs by influencing invertebrates of lower trophic levels that will use the algal structure as refuge, and, as a consequence, species of higher trophic levels that feed on these invertebrates.

The main aims of this study were to analyze the following: (i) the potential production of PUAs by four indigenous macroalgae (*Cystoseira compressa* (Esper) Gerloff and Nizamuddin, *Dictyopteris polypodioides* A.P. De Candolle J.V. Lamouroux, *Dictyota dichotoma* (Hudson) J.V.Lamouroux, *Ulva* cf. *lacinulata* (Kützing) Wittrock, and a non-indigenous species (*Sargassum muticum* (Yendo) Fensholt) and their effects on the microphyto- and meiobenthic communities; (ii) the relationship between the microphytobenthos and the meiobenthos present on the different macroalgal species, considering the structural complexity of the macroalgae and the potential role of aldehydes in regulating their interaction.

2. Materials and Methods

2.1. Study Area

This study was performed at two coastal sites in the north-western Adriatic Sea in the coastal area of Ancona, a city hosting a large commercial harbor (Figure 1). The first site was a semi-enclosed and shallow (mean depth 1.5 m) inlet called Piscinetta del Passetto (43°37′09″ N, 13°31′54″ E). The site, hereafter referred to as Passetto (PAS), was previously described [31]. The seaweed vegetation was characterized by the co-existence of many different macroalgae belonging to the class of Chlorophyta (e.g., *Ulva* cf. *lacinulata*, *Cladophora dalmatica*, *Cladophora laeteviren*), Ochrophyta (e.g., *Asperococcus fistulosus, Cystoseira compressa, Dictyota dichotoma, Dictyopteris polypodioides, Gongolaria barbata, Padina pavonica, Scytosiphon lomentaria*), and Rhodophyta (e.g., *Alsidium corallinum*, *Callithamnion corymbosum, Ceramium* spp., *Chondracanthus acicularis, Corallina berteroi, Gastroclonium clavatum, Gracilaria* spp., *Hypnea* spp., *Pterocladiella capillacea*), the latter being the most species-diverse [15]. The second study site (43°37′32″ N, 13°29′58″ E) was located along a wharf (Molo Nord) within the commercial harbor of Ancona and is hereafter referred to as Porto (POR). It is characterized by the presence of concrete blocks laid over a sandy bottom (Figure 1). The most common macroalgae present at this site include the following: Chlorophyta (e.g., *Bryopsis* spp., *Ulva* cf. *lacinulata*), a few native Ochrophyta (e.g., *Dictyota dichotoma*), and Rhodophyta (e.g., *Antithamnion cruciatum, Chondracanthus acicularis, Corallina berteroi, Gelidium* spp., *Pyropia elongata* and *Schottera nicaeensis*). Recent studies have documented in the harbor the presence of some non-indigenous species (NIS) such as *Aglaothamnion feldmanniae, Grateloupia turuturu, Melanothamnus japonicus, Polysiphonia morrowii*, and *Sargassum muticum* [15]; the occurrence of most of these has been known since 2009 [16].

For the study, five different macroalgal species were sampled (Figure S1): the native *Cystoseira compressa, Dictyopteris polypodioides, Dictyota dichotoma,* and *Ulva* cf. *lacinulata*, and the non-indigenous *Sargassum muticum*. These species were selected, in order to cover a wide range of morphological complexity and a variable PUAs production, as shown by previous studies [24,31], and to compare indigenous and invasive species.

2.2. Morphological Features of the Species Selected

Cystoseira compressa (Esper) Gerloff and Nizamuddin (Phaeophyceae) is a semi-perennial leathery macrophyte (i.e., most of the thallus is lost every year and the species persists in unfavorable seasons in the form of a small holdfast). The thallus consists of a basal callus arising one or a few short axes, issuing numerous branches arranged in a radial pattern, the length of which varied depending on the time of the year. At the time of full development (late spring–early summer), the thallus has a bushy habitat and may reach up to 1 m in height. This species has been reported to produce allelochemicals, in particular the short-chain polyunsaturated aldehyde hexadienal (C6:2) [31].

Figure 1. Sampling sites (© Google Earth).

Dictyota dichotoma (Hudson) J.V. Lamouroux (Phaeophyceae) is commonly found in the Mediterranean and Atlantic Seas and on rocks in calm places near the surface, and often associated with species of the closely related genus *Dictyopteris*. It has a ribbon-like corticated thallus, with regular dichotomous ramifications that end into bilobed and rounded apices. The color is olive green to yellowish brown. It measures up to 25 cm in height and tends to thrive in shallow, calm, and sheltered habitats. It has been documented by [31] that *D. dichotoma* is among the species of the class Phaeophyceae that produce long-chain PUAs, specifically eicosapentaenal (C20:5).

Ulva cf. *lacinulata* Wittrock (Ulvophyceae) is a leafy green seaweed with a thin thallus formed by two cell layers. The genus *Ulva* is taxonomically difficult and the alga used for this study corresponds morphologically to the species reported until recently in the Mediterranean as *Ulva rigida* C. Agardh. In [32], however, the authors highlighted a major taxonomic and nomenclatural problem concerning the identity of this species in the Mediterranean, suggesting that *Ulva lacinulata* is probably the correct identification for most Mediterranean specimens. Several studies have shown that algal specimens identified as *Ulva rigida* could produce a wide range of medium and long-chain aldehydes (nonatetraenal C9:4 and decatetraenal C10:4) [31,33].

Dictyopteris polypodioides (De Candolle) J.V. Lamouroux (Phaeophyceae) is a very common and abundant species mainly found in the Mediterranean Sea. The fronds are corticated, ribbon-like with a central midrib, with more or less irregular and proliferating edges; the height of thallus is 10–20 cm. *D. polypodioides* is known to produce phenolic compounds [4] which are probably involved in the defense against grazers (e.g., strong deterrent to the feeding of the amphipod). *D. polypodioides* was the major PUA producer among the species analyzed by Pezzolesi et al. [31], specifically for the production of long-chain PUAs such as tetradecapentaenal (C14:5) and hexadecatetraenal (C16:4).

Sargassum muticum (Yendo) Fensholt (Sargassaceae) is a large-sized leathery brown alga native to Japan; it is well-known as one of the most invasive seaweeds at global level. The occurrence of *S. muticum* in the harbor of Ancona has been known since 2009 [16]. This species is usually 1–3 m in length but can grow up to 16 m. The long, annual branches bear numerous small (<0.5 cm) round airbladders, making plants stand upright in the water or float on the surface, and small leaf-like branches. The alga has two distinct parts: the perennial, dark brown basal axes, and the lighter-colored annual primary laterals. The PUAs production for *S. muticum* is not yet known, but studies have reported the presence of phenolic compounds, particularly phlorotannins, making it unpalatable to grazers [34].

2.3. Sampling and Sample Processing

Sampling was carried out on 3 and 7 June 2021. Three macroalgal species were sampled in both sites: *Dictyota dichotoma* (DD), *Ulva* cf. *lacinulata* (UL), and *Dictyopteris polypodioides* (DP). Two species were sampled only in one site: *Cystoseira compressa* (CC) at Passetto and *Sargassum muticum* (SM) at Porto. The sampling time was chosen because in the study area this period corresponds to late spring. This is the only period in the year in which all the five species occur at the same time in their fully developed habitat, and therefore their thallus has the full morphological complexity.

Parts of the thalli (first 5–10 cm) for the branched species, and marginal fragments of *Ulva* cf. *lacinulata*, were sampled by snorkeling at a depth of about 0.5 m. For each macroalgal species, four replicates were collected using 50 mL polypropylene tubes (VWR International), avoiding the dispersion of the associated epiphytic organisms. Moreover, the surface temperature and salinity of the water column were measured at each site by a multiparameter water probe HQ30d (Hachl-Lange GmbH) and a refractometer Atago S-10, respectively.

Immediately after collection, the macroalgae were carried to the laboratory and processed to remove all associated benthic organisms, as described in Lenzo et al. [24]. Each tube containing the algal tips and their storage water was vigorously shaken to separate the macroalgae from the epiphytic microalgae and the meiofauna. Then, the tube was rinsed with filtered seawater and vigorously washed several times until epiphytic organisms were completely removed.

The total volume of washing seawater of each sample (approximately 150 mL) was measured and then divided into two aliquots, one for the microphytobenthos and the other for the meiofauna analyses. Aliquots for microphytobenthos analysis (about 75 mL) were fixed with Lugol and stored in 250 mL dark glass bottles. Aliquots for meiofauna analysis were sieved through a 1000 µm mesh and a 45 µm mesh. The meiofauna retained in the latter sieve was preserved in alcohol 70% and stored in a 50 ml tube until subsequent analyses.

2.4. Macroalgal Morphology

To determine whether the morphology of the macroalgae influenced the structure of the associated microphytobenthic and meiobenthic assemblages, different variables were measured for each thallus.

After removing the epiphytic organisms, the volume of each thallus was measured by placing the alga in a graduated cylinder containing water and determining the volume of water displaced. The thalli were then dried with absorbent paper and weighed to determine wet weight. Then, each frond was placed on a white surface with a reference scale and photographed using a digital camera Canon EOS 750D. Finally, the macroalgae samples were stored at −80 °C in new tubes.

Perimeter, area, and fractal dimensions (D), used as proxy of the habitat architecture [35], were measured processing the pictures using the program ImageJ v1.53u. The measure of fractal dimension (D) was based on the image using a method analogous to the grid method (boundary dimension) as previously proposed [36]. Moreover, for each sample volume, weight, and perimeter were standardized per area, according to the standardization made for microphytobenthos and meiofauna abundance.

2.5. Aldehydes (PUAs) Produced by Macroalgae

The extraction and quantification of PUAs produced by the macroalgae was carried out by gas chromatography–mass spectrometry (GC–MS) as previously described [31]. A portion of the apical part of the thallus (about 0.2–0.5 g f. wt.) was crushed with mortar and pestle in liquid nitrogen. The powder thus obtained was transferred into 10 mL tubes. Derivatization of the polyunsaturated aldehydes was performed with O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride solution (PFBHA HCl) and quantification

was based on the internal standard (i.e., benzaldehyde). All reagents were purchased from Sigma-Aldrich (Milan, Italy) and used without any further purification.

2.6. Microphytobenthic Assemblages

The quali-quantitative analysis of the microphytobenthos associated with the two macroalgae was performed using an inverted optical microscope (Zeiss Axiovert 100) at 320× and 200× magnification. Subsamples (5–10 mL) of epiphytic communities fixed with Lugol were settled in counting chambers after homogenization, according to the Utermöhl's sedimentation method [37,38]. Counting was performed in different ways. The microphytobenthos community was examined at 320× magnification on 30 random fields or 4–5 transects; then a counting at 200× of the organisms present on the whole sedimentation chamber was performed to obtain a correct evaluation of uncommon taxa. Orders and genera were identified based on various manuals and identification keys [39,40] or using data from the literature [41,42].

The identification of individuals was based exclusively on observable morphological characters (such as shape, size, number of chloroplasts); taxonomy and nomenclature for the microalgae recorded is based on AlgaeBase [43]. Abundance was expressed as number of cells per macroalgal area (cells/cm^2).

2.7. Meiobenthic Assemblages

Meiobenthic organisms of each sample were counted and identified to major taxa under a stereomicroscope (Nikon SMZ 1500). Since harpacticoid copepods were recognized as the most sensitive group to aldehydes [24], dead copepod harpacticoids and dead *nauplii*, recognizable by the empty exuviae, were counted separately. Furthermore, since PUAs may also impact harpacticoids reproduction causing a decreased egg viability, expelled egg sacs were also counted [24]. Abundance of meiobenthic organisms was expressed as Ind/cm^2.

2.8. Data Analysis

Due to the different macroalgal species sampled at the two sites, data were analyzed considering the site–macroalgae combinations as a single fixed factor with 8 levels. PERMANOVA tests [44,45] were performed to test for differences in variables related to macroalgae complexity, PUAs concentrations, number of taxa (S) and abundance (N) for microphytobenthos and meiobenthos, and in microphytobenthic and meiobenthic assemblages by a one-way design. PERMANOVA tests of univariate analyses were based on Euclidean distances of untransformed data, while tests for differences in microphytobenthic and meiobenthic community structures was based on Bray–Curtis similarity. Pairwise comparisons were performed when the main effect resulted as significant. When the number of unique permutations available was less than 100, asymptotic Monte Carlo *p*-values (P(MC)) were considered instead of permutational ones (P(perm)). All PERMANOVA analyses were performed using unrestricted permutation of the raw data and 9999 permutations.

Principal component analysis (PCA) ordination, performed on normalized data, was used to display the relationship between morphological measures taken for each macroalgal sample, and to identify the most important variables that differentiate the habitats that they provide to microphyto and meiobenthic assemblages. Before performing the PCA analysis, the distribution and the possible correlation among variables were evaluated by draftsman plot (by applying a $|r| < 0.95$ cut-off).

Community data of microphytobenthos and meiobenthos were transformed considering the results obtained by shade plots. These routines provide a simple visual representation of abundance matrices from multivariate species assemblage, which guide the choice of the best transformation of quantitative data [46]. Microphytobenthic abundance data were fourth root transformed, while meiofauna abundance data were transformed by square root. The community structure of each assemblage (microphytobenthos and meiofauna) was analyzed by non-metric multidimensional scaling (nMDS) based on Bray–Curtis similarity.

Taxa that mostly contributed to the dissimilarity/similarity among/within macroalgal species were identified using the SIMPER analysis (60% cut-off for microphytobenthos and 70% cut-off for meiobenthos) [47].

Multivariate multiple regression, using a distance-based linear model (DistLM) procedure (in PERMANOVA+; [48]) was used to test for relationships between the set of macroalgal morphological traits and PUAs and microphytobenthic assemblages. To analyze the relationships between macroalgal complexity, PUAs, and microphytobenthic abundance with meiofauna communities, DistLM was performed on two different meiofauna matrices: all taxa and all taxa with the addition of harpacticoid and *nauplii* dead. The "BEST" procedure and Akaike Information Criterion (AICc, was used as selection criterion. Distance-based Redundancy Analysis (dbRDA) plot was used to visualize DistLM results.

Finally, to compare the response of microphytobenthic and meiobenthic assemblages to site-macroalgae, a RELATE analysis was performed.

The significance level was set at 0.05 (5%) for all tests. All analyses were conducted with PRIMER v7 [49] with the PERMANOVA + add on [48].

3. Results

Seawater temperature and salinity measured in the seawater during the samplings were 21.7 °C and 37 ‰ in Passetto and 20.1 °C and 38 ‰ in Porto, respectively.

3.1. Macroalgal Morphology

Overall, five measures were taken to evaluate the morphology of the macroalgae and three ratios were calculated to estimate the complexity of the apical parts of each species (Table 1).

Table 1. Mean value (±SE; n = 4) of the variables measured in Passetto (PAS) and Porto (POR) on each macroalga: *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva* cf. *lacinulata* (UL), and *Dictyopteris polypodioides* (DP) and *Sargassum muticum* (SM).

Site–Macroalgae	Weight (g)	Volume (mL)	Area (cm^2)	Perimeter (cm)	D	Weight/Area	Perimeter/Area	Volume/Area
PAS-CC1	0.80 ± 0.07	1.05 ± 0.06	9.74 ± 0.77	185.10 ± 14.39	1.74 ± 0.01	0.08 ± 0.002	19.03 ± 0.57	0.11 ± 0.00
PAS-DD1	0.13 ± 0.01	0.28 ± 0.09	5.16 ± 0.39	68.09 ± 9.30	1.76 ± 0.01	0.03 ± 0.002	13.06 ± 0.86	0.05 ± 0.01
PAS-UL1	0.45 ± 0.03	1.23 ± 0.09	25.68 ± 2.23	49.70 ± 4.70	1.94 ± 0.00	0.02 ± 0.000	1.99 ± 0.26	0.05 ± 0.00
PAS-DP1	0.34 ± 0.02	0.93 ± 0.05	15.71 ± 1.26	56.98 ± 2.76	1.84 ± 0.01	0.02 ± 0.001	3.69 ± 0.32	0.06 ± 0.01
POR-SM1	0.66 ± 0.08	1.18 ± 0.12	11.12 ± 1.28	257.45 ± 29.51	1.70 ± 0.02	0.06 ± 0.003	23.22 ± 1.06	0.11 ± 0.01
POR-DD1	0.32 ± 0.01	0.50 ± 0.00	19.59 ± 0.52	150.48 ± 7.08	1.78 ± 0.01	0.02 ± 0.001	7.68 ± 0.30	0.03 ± 0.00
POR-UL1	0.28 ± 0.06	1.38 ± 0.24	35.84 ± 7.70	66.42 ± 13.77	1.92 ± 0.00	0.01 ± 0.000	1.88 ± 0.16	0.04 ± 0.01
POR-DP1	0.36 ± 0.06	0.93 ± 0.22	18.75 ± 2.98	68.40 ± 11.78	1.88 ± 0.01	0.02 ± 0.000	3.63 ± 0.06	0.05 ± 0.01

The complexity of macroalgae varied among the different analyzed species. *Ulva* cf. *lacinulata* had the highest fractal dimension (D) in both sites, followed by *Dictyopteris polypodioides*, while the lowest values were measured in *Sargassum muticum* and *Cystoseira compressa.* An almost opposite trend was observed for the perimeter and the other measures standardized per area. The PCA analysis has been performed on D and ratios between weight, volume, and perimeter to area, in order to be consistent with the standardization carried out for microphytobenthos and meiofauna abundance.

The first two components accounted for 95.2% of the total variance (Figure 2). Variability along the PC1 axis was mainly explained, from left to right, by a decrease of weight/area and perimeter/area ratios while variability along the PC2 axis was mainly explained, from top to bottom, by an increase of fractal (D) (Tables 1 and S1). Accordingly, the samples were disposed more or less regularly along these gradients, with samples of *C. compressa* in Passetto and *S. muticum* in Porto grouped together in the left side of the PC1, and, in the right side, all the other macroalgae collected in both sites. The second axis detected morphological variation among macroalgae, that formed clusters depending on the macroalgal species (Figure 2). This pattern was confirmed by PERMANOVA results that showed signif-

icant differences among the site–macroalgae factor (PseudoF = 50.86, $p = 0.0001$; Table S2). Post hoc comparisons among macroalgal species within each site and between sites for each species present either in Passetto or Porto confirmed the morphological differences among macroalgae in each site, except for POR-UL vs POR-DP. Instead, morphology of *Ulva* cf. *lacinulata* and *Dictyopteris polypodioides* in the two sites did not differ, suggesting morphological coherence despite the different sites.

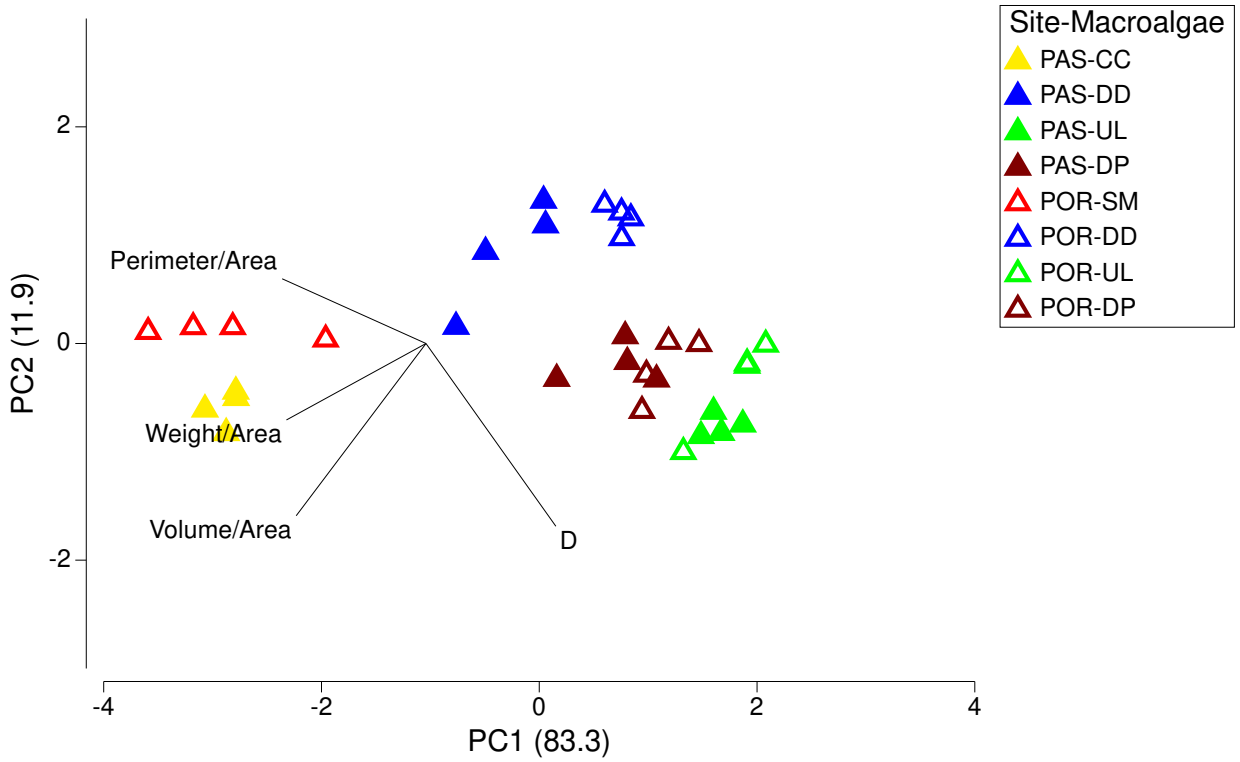

Figure 2. Principal component analysis (PCA) ordination carried out on untransformed macroalgal morphological data. PCA was performed on the normalized measures and measure ratios taken in Passetto (PAS) and Porto (POR) on each macroalga: *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva* cf. *lacinulata* (UL), and *Dictyopteris polypodioides* (DP) and *Sargassum muticum* (SM).

3.2. Unsaturated Aldehydes (PUAs) Produced by Macroalgae

Quantitative and qualitative concentrations of the main aldehydes produced by the macroalgae sampled in Passetto and Porto obtained through GC-MS analysis highlighted significant differences among macroalgae and sites. The total average concentrations of PUAs detected in the macroalgae sampled in the two sites were shown in Figure 3a.

In the Passetto site, the highest aldehyde production was detected in PAS-DP, with a concentration of 1032.8 nmol/g. In PAS-DD, the total amount resulted as 69.7 nmol/g, while in PAS-UL and PAS-CC, PUAs concentrations were very low (<12 nmol/g). In the Porto site, POR-UL was the major PUAs producer (865.2 nmol/g), while PUAs concentrations in POR-SM, POR-DD, and POR-DP were low and very similar, ranging between 43 and 47 nmol/g. This pattern was supported by the statistical analysis (PERMANOVA, PseudoF = 14.39; $p = 0.0003$; Table S3). In Passetto, a significantly higher concentration was measured in PAS-DP compared to the concentrations measured in the other algae; to a lesser extent a relatively higher concentration occurred in PAS-DD than in PAS-UL and PAS-CC. In Porto, post hoc results highlighted the significantly higher concentration in POR-UL than in all other macroalgae that did not show significant differences. Pairwise comparisons made between sites for each corresponding macroalga evidenced a significantly higher concentration in POR-UL than in PAS-UL and a higher concentration in PAS-DP than in POR-DP (Table S3).

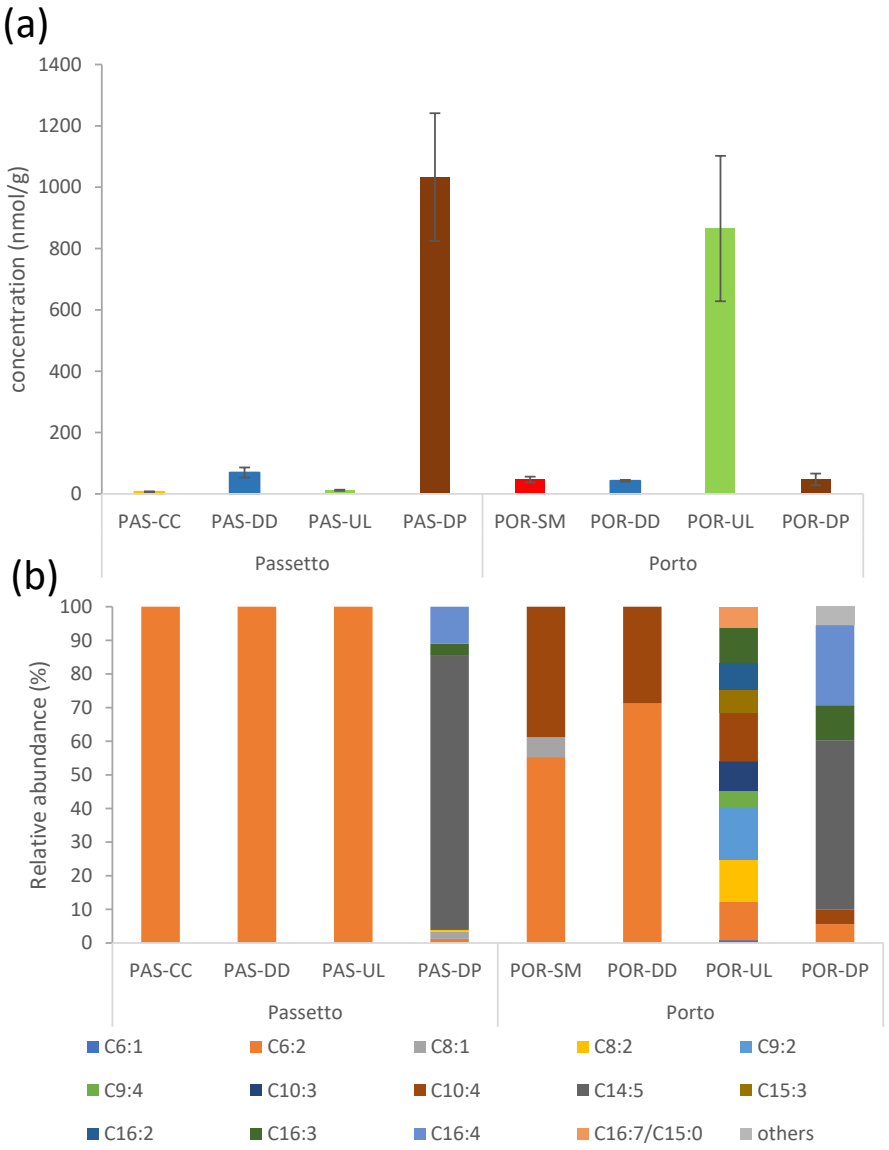

Figure 3. Mean (±SE; n = 4) of (**a**) total concentration (nmol/g) and (**b**) relative abundance (%) of polyunsaturated aldehydes in the different macroalgae collected in Passetto (PAS) and Porto (POR): *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva* cf. *lacinulata* (UL), *Dictyopteris polypodioides* (DP) and *Sargassum muticum* (SM).

From a qualitative point of view, PUAs relative abundance measured in each macroalga in the two sites showed high variability (Figure 3b). The small-chained compound hexadienal (C6:2) was the most abundant aldehyde in several macroalgal species (i.e., POR-SM and POR-DD), and reported relative abundances up to 100% in PAS-CC, PAS-DD, and PAS-UL. Conversely, PASS-DP highlighted the production of long-chained aldehydes, such as tetradecapentaenal (14:5) and hexadecatetraenal (16:4) which were the most abundant (about 82 and 11%, respectively). In Porto, decatetraenal (C10:4) was among the main aldehydes in several species, with relative abundances up to 29% and 39% in POR-DD and POR-SM, respectively. As for POR-DP, the PUAs profile was similar to the one detected for PAS-DP, although C14:5 was less abundant (50%), while relative abundances of C16 aldehydes were higher and resulted in 10% and 24% for hexadecatrienal (C16:3) and C16:4, respectively. POR-UL showed a PUAs profile completely different from the one reported for PAS-UL, characterized by a variety of small- (i.e., C6:2, octadienal C8:2), medium- (i.e., nonadienal C9:2, C10:4), and long-chained (i.e., C16:3) compounds, all present at relatively low abundances (about 10–15%).

3.3. Microphytobenthic Assemblages

Species belonging to at least 27 microalgal genera were identified, and some diatom species remained unknown (namely undetermined pennate or centric diatoms) (Table S4). Generally, the number of taxa (S) varied significantly (PERMANOVA results: PseudoF = 10.50; $p < 0.0001$; Table S5) among site–macroalgae and a higher number of taxa was observed on the macroalgae collected in Passetto (ranging between 14.25 and 17.75) compared to Porto (ranging between 8.5 and 12.25). Post hoc results showed a significantly higher number of taxa on PAS-UL and PAS-DD in comparisons to POR-UL and POR-DD, respectively, while in Porto, a significantly lower number of taxa resulted on POR-DP with respect to POR-SM and POR-DD (Figure 4a; Table S5). Results of PERMANOVA on the total microphytobenthos abundance showed a significant effect of site–macroalgae (PseudoF = 4.613; $p = 0.0026$; Table S5). Post hoc comparisons indicated that the highest abundances for each site, observed on PAS-DD (46,412 cell/cm^2) and POR-SM (45,080 cell/cm^2), were significantly higher only compared to PAS-UL and POR-UL, respectively (ST-3.4). Conversely, in PAS-UL and POR-UL, microphytobenthos abundance was lower compared to other samples (i.e., PAS-CC; and POR-DD and POR-DP), reporting values of 11,606 and 2480 cell/cm^2, respectively. Microphytobenthos abundances on POR-DD and POR-DP were 32,814 and 14,488 cell/cm^2 and resulted as significantly different (Figure 4b; Table S5).

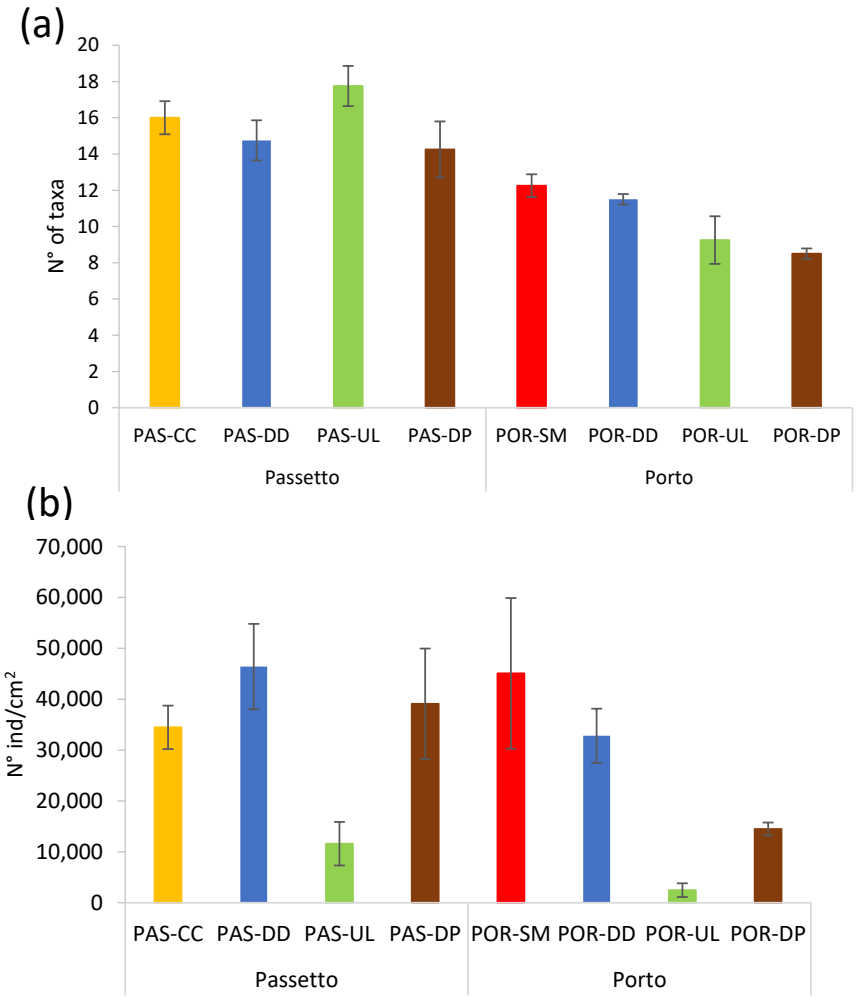

Figure 4. Mean (±SE; n = 4) of (**a**) number of taxa and (**b**) total abundance of the microphytobenthic community measured in Passetto (PAS) and Porto (POR) on each macroalga: *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva* cf. *lacinulata* (UL), *Dictyopteris polypodioides* (DP), and *Sargassum muticum* (SM).

Globally, the taxonomic composition of the microphytobenthos communities associated to the different macroalgae in both sites was dominated by diatoms (Figure 5), although representatives of several algal classes were reported (Bacillariophyceae, Mediophyceae, Xantophyceae, Dinophyceae). Specifically, in PASS-CC, the relative abundance of Mediophyceae resulted higher than in all other samples due to the abundance of *Leptocylindrus* spp. (46%), while Bacillariophyceae were generally more abundant (about 80–95%) and Dinophyceae below 5% except for POR-UL (21%) in all other samples. *Cocconeis* spp. resulted more abundant in macroalgae collected in Passetto (up to 24% in PAS-DP), while *Navicula* spp. were present on each macroalgal species in the two sites, with the lowest relative abundance in POR-SM (14%) and the highest in PASS-DP (48%). *Nitzschia* spp. and *Pseudo-nitzschia* spp. mainly characterized the microphytobenthic community in POR-SM with relative abundances of 40% and 4%, respectively. Some dinoflagellate species belonging to the genera *Prorocentrum* and *Amphidinium*, but also to some known to have a planktonic behavior (i.e., *Alexandrium* spp.), were reported, although at low abundances. Macroalgae collected in Porto, and in particular POR-DD, were characterized by a high abundance of pennate diatoms whose identification remained undetermined (namely undetermined pennate diatoms). POR-UL microphytobenthic assemblage resulted less characterized by diatoms as, together with dinoflagellates, also small cells (<20 µm) were abundant (49%). These cells were characterized by observable morphological characters ascribable to species belonging to the Cryptophyceae, although further in-depth investigation would be needed to better clarify their taxonomic identity.

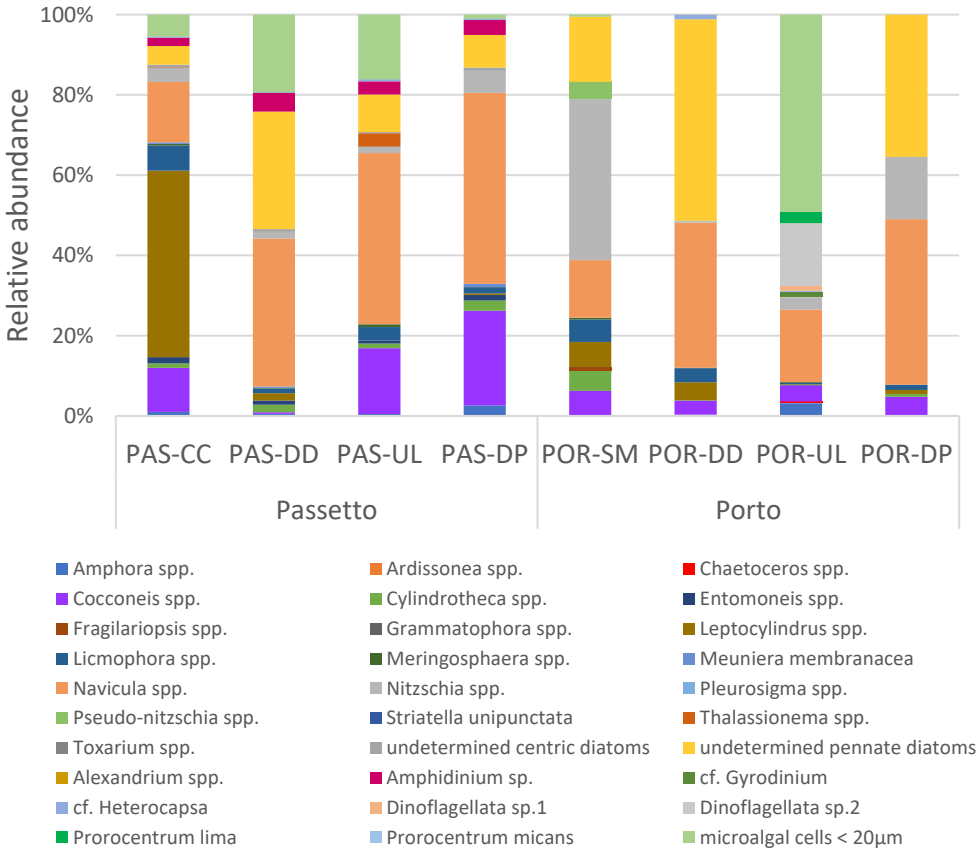

Figure 5. Taxonomic composition of the microphytobenthos community in Passetto (PAS) and Porto (POR) on each macroalga: *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva* cf. *lacinulata* (UL), *Dictyopteris polypodioides* (DP) and *Sargassum muticum* (SM).

The nMDS analysis of the microphytobenthos communities showed two distinct groups, identified by samples collected at the two different sites, with POR-UL samples slightly separated and showing high dispersion (Figure 6). PERMANOVA carried out on

taxonomic structure showed significant differences among site–macroalgae (PseudoF = 8.59, $p = 0.0001$; Table S6). According to the results of the pairwise comparisons, the community structure was significantly different on the same macroalgal species collected in the two sites. Within each site, the differences among macroalgae species were all significant, except for PAS-DP which was not significantly different from any of the other species sampled at Passetto.

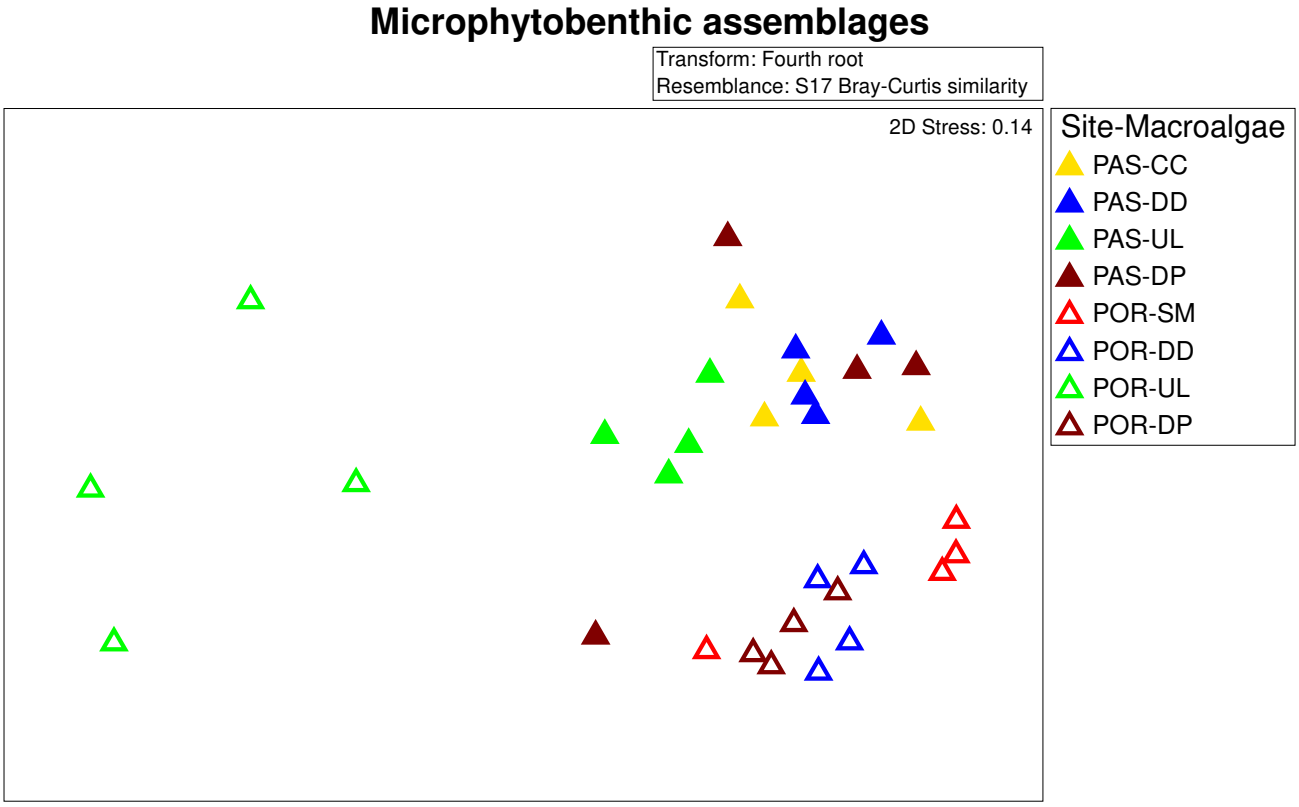

Figure 6. nMDS ordination of a sample of microphytobenthic assemblages based on fourth-root transformed abundance (cell/cm^2) and Bray–Curtis similarity in Passetto (PAS) and Porto (POR) associated with each macroalga: *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva* cf. *lacinulata* (UL), *Dictyopteris polypodioides* (DP), and *Sargassum muticum* (SM).

SIMPER analysis revealed that average dissimilarities between macroalgae sampled in Passetto ranged from 30.19% between PAS-C and PAS-DD to 40.37% between PAS-UL and PAS-DP. In general, dissimilarities were largely due to the variations in abundance of some diatoms (e.g., species belonging to the genera *Leptocylindrus* and *Cocconeis*, or undetermined pennate diatoms), without a clear pattern. On the other hand, in Porto, average dissimilarities between macroalgae were higher than in Passetto, ranging between 31.50% (POR-DD and POR-DP) and 69.46% (POR-DD and POR-UL), and mainly due to abundance variation of *Navicula* spp., *Pseudo-nitzschia* spp., *Nitzschia* spp., or undetermined pennate diatoms which resulted generally abundant in POR-DD and POR-SM (Table S7).

The comparison among microphytobenthos communities found on the same macroalga in the two sites showed the highest dissimilarity between PAS-UL and POR-UL (58.36%) and was mainly due to the higher abundance of undetermined pennate diatoms, and species belonging to the genera *Navicula*, *Amphidinium*, and *Cocconeis* in PAS-UL (Table S7).

3.4. Meiobenthic Assemblages

In total 13 major meiobenthic taxa were identified across samples (Figure 7; Table S8). Copepod *nauplii* have been considered a taxon, separate from later copepod stages [24]. Globally, *nauplii* and harpacticoids dominated in both sites on all macroalgal species. In

Passetto on PAS-DP, a relatively higher diversity of taxa was found where amphipods, isopods, and gastropods represent a small percentage of the total. Moreover, in Passetto, all the four macroalgae hosted a greater variability of taxa in comparison with those hosted on macroalgae in Porto, where more than 70% was represented by copepod *nauplii* followed by adult harpacticoids. (Figure 7).

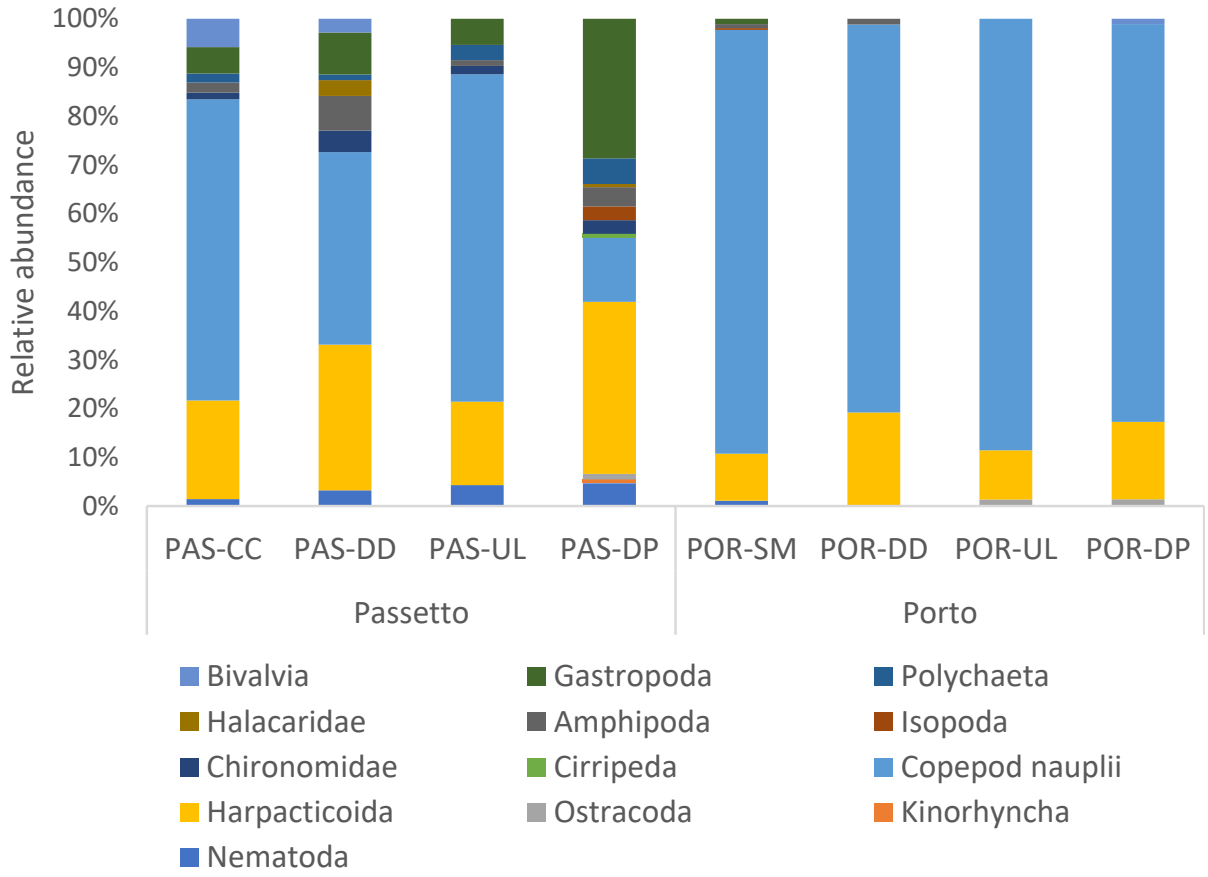

Figure 7. Relative abundance of major meiobenthic taxa in Passetto (PAS) and Porto (POR) on each macroalga: *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva* cf. *lacinulata* (UL), *Dictyopteris polypodioides* (DP), and *Sargassum muticum* (SM).

The number of major meiobenthic taxa (S) varied significantly (PERMANOVA results: Pseudo-F = 13.23; p = 0.0001; Table S9) among site–macroalgae and the highest value was found on PAS-DP (8.5 ± 0.96) and the lowest on POR-UL (2 ± 0.41) (Figure 8a). In Passetto, pairwise comparisons showed a significant highest number of taxa on PAS-DP in comparisons with all the other three macroalgae, while in Porto the number of taxa did not show significant differences (Table S9). The number of taxa on the same macroalga in the two sites resulted always significantly higher in Passetto. Results of PERMANOVA on the total abundance (N) showed a significant effect of site–macroalgae (Pseudo F = 2.15; p = 0.049; Table S9) (Figure 8b). Post hoc comparisons indicated that the abundance on PAS-DP resulted higher than PAS-UL, while in Porto a higher abundance occurred on POR-SM in comparison with POR-DD and POR-UL (Table S9). Comparisons between the same macroalga in the two sites showed significantly higher abundance in PAS-DP than POR-DP.

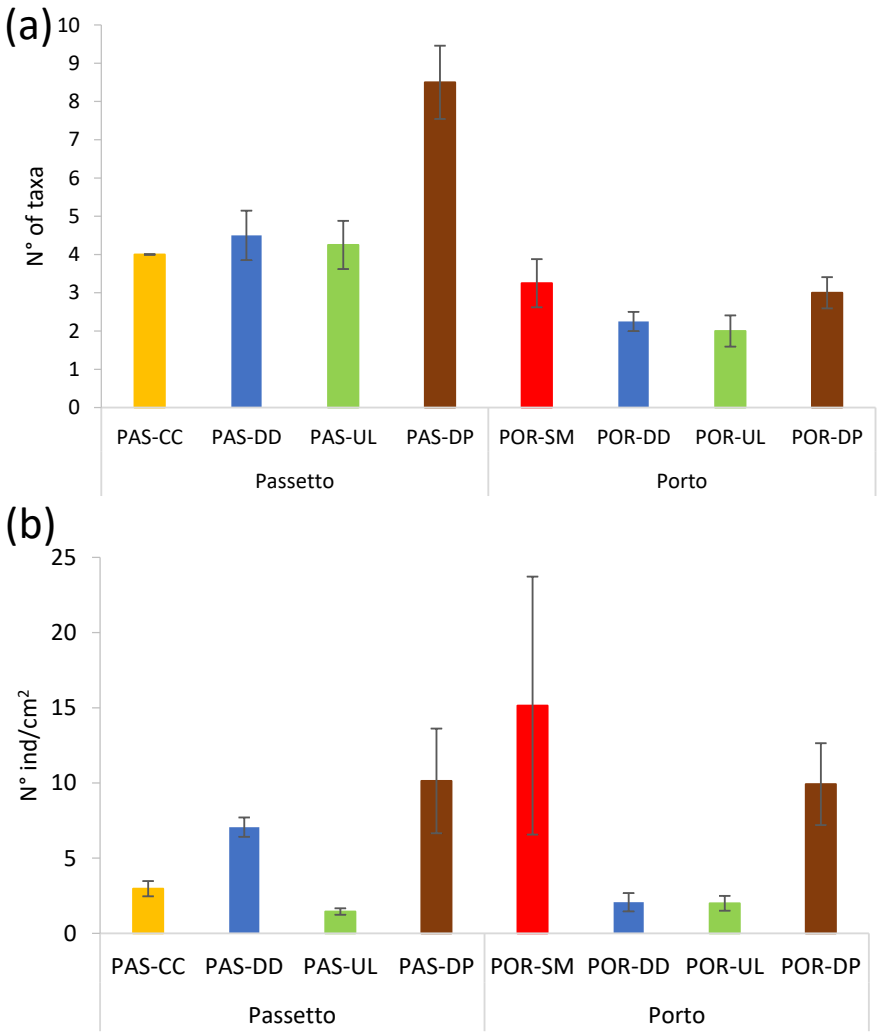

Figure 8. Mean (±SE; n = 4) of (**a**) number of taxa and (**b**) total abundance of the meiobenthic community in Passetto (PAS) and Porto (POR) on each macroalga: *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva* cf. *lacinulata* (UL), *Dictyopteris polypodioides* (DP), and *Sargassum muticum* (SM).

The nMDS plot showed the meiofauna assemblages present in the Passetto relatively separated from those in Porto but changes of meiobenthic communities depended on samples belonging to the different macroalgae (Figure 9).

PERMANOVA carried out on taxonomic structure (PseudoF = 4.58, $p = 0.0001$; Table S10) showed significant differences among site–macroalgae. In Passetto, the significantly different assemblages resulted between PAS-CC vs PAS-DP, PAS-DD vs. PAS-UL and PAS-UL vs. PAS-DP, while in Porto significantly different assemblages occurred between POR-SM vs. POR-UL, POR-DD vs. POR-DP and POR-UL vs. POR-DP. Moreover, the community structure was significantly different on corresponding macroalga in the two sites. (Table S10).

SIMPER analysis revealed that average dissimilarities between macroalgae in Passetto ranged from 60.80% between PAS-UL and PAS-DP to 37.61% between PAS-CC and PAS-UL. In general, dissimilarities between macroalgae were largely due to the variations in abundance of almost all identified taxa, without a clear pattern. On the other hand, in Porto, average dissimilarities between macroalgae were lower, ranging from 48.98% between POR-SM and POR-UL, to 23.59% between POR-DD and POR-UL and were mainly due to abundance variation of Copepod *nauplii* and harpacticoids that, in combination contributed to about 62–84% of total dissimilarities (Table S11).

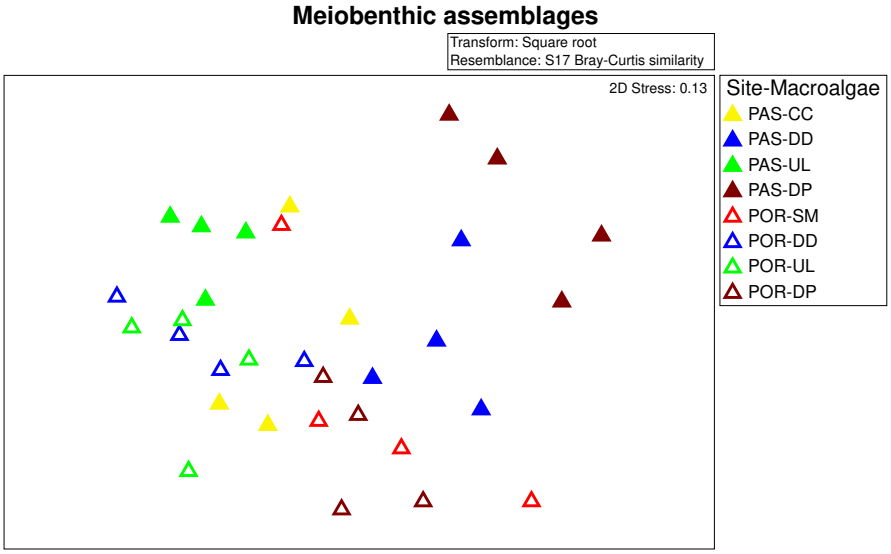

Figure 9. nMDS ordination of sample of meiobenthic assemblages based on square-root transformed abundance (N/cm^2) and Bray–Curtis similarity in Passetto (PAS) and Porto (Por) associated with each macroalga: *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva* cf. *lacinulata* (UL), *Dictyopteris polypodioides* (DP), and *Sargassum muticum* (SM).

Comparing meiobenthic communities on the same macroalga in the two sites, the highest dissimilarity was between PAS-DP and POR-DP (60.34%) and was due to the higher abundance of copepod *nauplii* on POR-DP and the absence of the other taxa present only on PAS-DP (Table S11).

Furthermore, considering the possible effect of PUA production on copepods, the number of dead harpacticoids (copepodites and adults), dead copepod *nauplii*, and expelled egg sacs were also counted (Table 2). The highest number of dead *nauplii* and adults were found on DP in Passetto. In general, dead copepod *nauplii* were more present on all macroalgae sampled in Porto, especially on POR-SM, while dead adult harpacticoids occurred on all macroalgae in both sites. A different result was obtained for expelled egg sacs that were high on PAS-CC (8.25 egg sac/cm^2) and PAS-DD (16.02 egg sacs/cm^2), and to a lesser extent on POR-SM (4.03 egg sacs/cm^2).

Table 2. Mean value (±SE; n = 4) of the abundance (Ind/cm^2) of dead copepod *nauplii*, dead harpacticoids and expelled egg sacs in Passetto (PAS) and Porto (POR) on each macroalga: *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva* cf. *lacinulata* (UL), *Dictyopteris polypodioides* (DP), and *Sargassum muticum* (SM).

	Dead Copepod *nauplii*	**Dead Harpacticoids**	**Expelled Egg Sacs**
PAS-CC		0.06 ± 0.06	8.25 ± 0.06
PAS-DD		0.10 ± 0.06	16.02 ± 0.06
PAS-UL		0.05 ± 0.02	1.11 ± 0.02
PAS-DP	0.31 ± 0.17	0.37 ± 0.12	2.84 ± 0.12
POR-SM	0.11 ± 0.11	0.05 ± 0.03	4.03 ± 0.03
POR-DD	0.03 ± 0.03	0.03 ± 0.02	0.08 ± 0.02
POR-UL	0.03 ± 0.02	0.01 ± 0.01	0.11 ± 0.01
POR-DP	0.08 ± 0.04	0.09 ± 0.03	0.91 ± 0.03

3.5. Relationship between the Microphytobenthos and Meiofauna Assemblages with Macroalgae Complexity and PUAs Production

To evaluate the relationships between the community structure of both microphytobenthos and meiofauna with macroalgae complexity and PUAs production, D, weight/area, perimeter/area, and volume/area and PUAs tot (nmol/g) were entered in DistLM analysis.

The results of DistLM carried out to analyze the relationship between PUAs and macroalgal variables and the microphytobenthos were shown in Table 3a. The marginal test showed that all the analyzed variables had a significant relationship with the assemblages when considered alone and the fractal D explained nearly 15% of the variability in the data. The best selection included three variables: D, perimeter/area and PUAs tot (nmol/g) that explained 30% of variance in microphytobenthos structure.

Table 3. Results from the DistLM analysis of the influence of measure of macroalgae complexity and PUAs concentration on (a) microphytobenthic assemblage structure; (b) meiobenthic assemblage structure; (c) meiobenthic assemblage structure considering also total abundance of microphytobenthos (abund-microphyto); and (d) meiobenthic assemblage structure considering also dead harpacticoids, dead copepod nauplii end expelled egg sacs. Statistically significant *p*-values are in bold.

(a)	**Variable**	**Pseudo-F**	***P***	**Prop**
Marginal test	D	5.441	**0.0001**	0.154
	Weight/Area	3.850	**0.0033**	0.114
	Perimeter/Area	4.288	**0.0011**	0.125
	Volume/Area	3.184	**0.0072**	0.096
	PUAs (nmol/g)	3.745	**0.0061**	0.111
	Model	AICc	R^2	RSS
Best solution		222.0300	0.30	24533
	D; Perimeter/Area; PUAs			
(b)	Variable	Pseudo-F	*P*	Prop
Marginal test	D	1.598	0.1684	0.051
	Weight/Area	0.640	0.6566	0.021
	Perimeter/Area	0.942	0.4193	0.030
	Volume/Area	0.706	0.6045	0.023
	PUAs (nmol/g)	1.438	0.2063	0.046
	Model	AICc	R^2	RSS
Best solution		227.72	0.05	34342
	D			
(c)	Variable	Pseudo-F	*P*	Prop
Marginal test	D	1.598	0.165	0.051
	Weight/Area	0.640	0.648	0.021
	Perimeter/Area	0.942	0.427	0.030
	Volume/Area	0.706	0.593	0.023
	PUAs (nmol/g)	1.438	0.204	0.046
	Abund Microphyto	3.610	**0.010**	0.107
	Model	AICc	R^2	RSS
Best solution		225.60	0.18	29777
	PUAs; Abund Microphyto			
(d)	Variable	Pseudo-F	*P*	Prop
	D	2.153	0.0561	0.067
	Weight/Area	1.764	0.1056	0.056
	Perimeter/Area	1.542	0.1617	0.049
	Volume/Area	1.172	0.3007	0.038
	PUAs (nmol/g)	2.226	**0.0485**	0.069
	Model	AICc	R^2	RSS
Best solution		233.70	0.16	38351
	D; PUAs			

The DistLM carried out to analyze the relationship between PUAs and macroalgal variables and the meiofauna assemblages, showed that none of the variables were significant predictors and the best selection of analyzed variables included only D (Table 3b), but explained only 5% of variance in the meiobenthic community structure. The analysis was repeated adding the microphytobenthos abundance to the other variables considered

in the previous analysis. Results (Table 3c) showed that the only significant predictor resulted total abundance of microphytobenthos, which however explained only the 11% of variance in meiobenthic assemblage structure (Table 3c). The best selection of analyzed variables included PUAs tot and total abundance of microphytobenthos that, however, explained only 18% of variance in meiobenthic community structure. Finally, the DistLM was performed between PUAs and the macroalgal variables and the meiofauna, including also the number of dead copepod nauplii, dead harpacticoids, and expelled egg sacs. In this analysis, PUAs concentration resulted in the only significant predictor which however explained only 7% of variance in meiobenthic assemblage structure and the best solution explaining overall variation of assemblages included only D and PUAs that all together explained a very low variance (16%) in meiofauna (Table 3d).

The marginal test reports Pseudo-F and *p* value and explained the variation for each variable, and the best solution reports the selection of variables that generate the highest explained variation at the lower AICc value.

The dbRDA ordination of microphytobenthic assemblages with superimposed explanatory variables (Figure 10a) showed that the first axis detected a variation in community structure among algae with different D and PUAs production, while the second axis highlighted the variability among samples between sites and is mainly driven by perimeter/area ratio (Figure 10a). The dbRDA ordination of meiobenthic assemblages, with also dead harpacticoids, dead nauplii, and expelled egg sacs with superimposed explanatory variables showed that D and PUAs were the variables that influence the communities, but in the opposite way (Figure 10b).

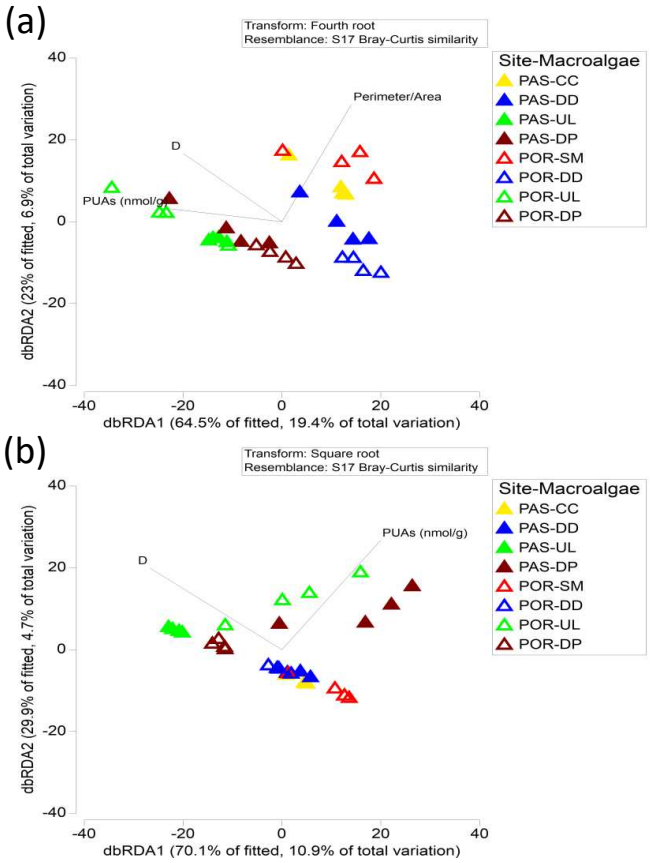

Figure 10. Distance-based redundancy analysis (dbRDA) of (**a**) microphytobenthos samples and (**b**) meiofauna samples including dead copepod nauplii, dead harpacticoids, and expelled egg sacs in Passetto (PAS) and Porto (POR) on each macroalga: *Cystoseira compressa* (CC), Dictyota dichotoma (DD), *Ulva* cf. *lacinulata* (UL), *Dictyopteris polypodioides* (DP), and *Sargassum muticum* (SM). Normalized predictor variables (based on distLM analysis in Table 3) are overlaid.

The different response of microphytobenthic and meiobenthic assemblages to macroalgae and PUA production in the two sites was confirmed by the RELATE result that showed a not significant correlation between the two multivariate matrices (Rho = 0.13; $p = 0.08$).

4. Discussion

Differences in the morphological complexity of five macroalgal species, including the introduced species *Sargassum muticum*, were explored in this study. Different variables estimating the structural complexity of macrophytes have been considered in different studies [50]. Most studies have used variables related to the amount of habitat provided (i.e., biomass, surface area, or volume), rather than true complexity measures. On the other hand, variables such as perimeter and fractal dimensions (D) are more suitable to provide a proxy of the habitat architecture [35]. In this study we decided to use volume/area ratio (for which high values indicate a more three-dimensional morphology), weight/area ratio (which provides a measure of available habitat standardized per surficial area), and perimeter/area ratio (to compare thalli that do not present a regular geometrical shape).

Multivariate PCA results allowed to discern the main variables that described the complexity of the macroalgae in the two sites and did not seem to be redundant. Moreover, all the measures collected for the same macroalga were consistently comparable in the two sites, suggesting that at the time of sampling each species exhibits consistent morphological characteristics. The macroalgae were sampled within one week at the same time of the year, in order to guarantee that comparisons between sites and species would not be affected by seasonal variation, which strongly influences the morphological structure of the species considered [51,52].

High values of all ratios (weight, perimeter, or volume to area) were calculated for *S. muticum* and *C. compressa*, indicating that these species were more complex compared to the others, while D values resulted as high in *U.* cf. *lacinulata* and *D. polypodioides* in both sites. The low D values measured for *S. muticum* and *C. compressa* seem to contradict the notion that fractal of macroalgae increases with the complexity [53]. Although efforts were made to obtain two-dimensional samples by breaking up the thalli, recent studies remarked the limitation in using a two-dimensional photographs to estimate the true complexity found in naturally suspended plants and recognized that the three-dimensional architecture typical of a plant is not completely represented by a two-dimensional image [54].

To understand if the production of bioactive compounds could influence the microphyto and meiobenthic communities associated with the different macroalgae, we focused on a class of compounds well known for having allelopathic effects, i.e., polyunsaturated aldehydes (PUAs). The production of a variety of biomolecules by algae is widely recognized [55]. In particular, macroalgae are among the main producers of polyunsaturated fatty acids (PUFAs), such as linoleic acid, which are not only essential components of membranes but can be involved in the regulation of physiological processes, serving also as precursors in the biosynthesis of structurally diverse oxylipins, including PUAs [53]. Recent studies [24,31] highlighted a great difference in the profile of aldehydes produced by different macroalgae, including species belonging to the same family, especially in relation to the length of their carbon chains and number of unsaturations. In particular, some species such as *D. polypodioides* and *U.* cf. *lacinulata*, produce higher amounts of PUAs compared to other macroalgae (e.g., *C. compressa*, *Padina pavonica, Gracilaria* sp.) [31]. The short-chain PUA C6:2 was the main compound in several species (e.g., *Ceramium ciliatum, Padina pavonica, Cystoseira compressa*), while medium-chain PUAs (i.e., C9:4 and C10:4) were dominant in *Ulva* cf. *rigida* (now referred to as *U.* cf. *lacinulata*) [31]. For long-chain aldehydes (i.e., C20:5 C14:5, C16:3 and C16:4.), a higher production is known for *Dictyota dichotoma* and *Dictyopteris polypodioides* [24,31]. These results were confirmed in the present study, where the same algal species previously analyzed [31] yielded a similar PUAs profile, regardless of the site. In fact, the macroalgae that showed the highest production of long and medium-chain PUAs were *D. polypodioides*, which produced C14:5 and C16:4, and *D. dichotoma*, which produced C10:4, together with *S. muticum*. The short-chain PUA C6:2

was produced by several species such as *C. compressa*, *U.*cf. *lacinulata, D. dichotoma,* and *S. muticum*.

From a quantitative point of view, *D. polypodioides* showed the highest concentration of PUA (1032.8 nmol/g), especially in the Passetto site, similarly to the results of a previous study [24], where concentrations of 225.5 µg/g ww (corresponding to 2127.3 nmol/g) were reported. These results confirmed that PUA profiles can be used as a fingerprint of each algal species, as the same compounds were consistently detected regardless of the sampling site. Conversely, their relative and total amount may vary depending on environmental conditions or morphotypes [31,33].

Differences between specimens from different sites were detected for *Ulva* cf. *lacinulata*, in which high qualitatively and quantitatively different profile PUAs were observed in samples collected in Porto and in Passetto. Specifically, *U*. cf. *lacinulata* in the Passetto produced only the short-chain compound C6:2 at a low concentration (11.8 nmol/g), while in the Porto it produced several compounds at high concentrations, including short-chain aldehydes, such as C6:2 (100.6 nmol/g), medium chain PUAs such as C8:2, C9:2, C10:4 (110.9 nmol/g, 137.5 nmol/g, and 128.8 nmol/g, respectively), and long chain ones such as C16.3 (94.0 nmol/g). Middle and long-chain PUAs had already been described for *Ulva* spp., specifically C7:2, C10:2, and C10:3 [33], as well as C16:3, C17:2, and C17:3 in *U. pertusa* [56,57]. The fact that PUA profiles represent a fingerprint for each species leads to the hypothesis that the *Ulva* samples collected in the two sites may belong to different species. Species of *Ulva* (Ulvophyceae, Chlorophyta) are among the most common algae in intertidal environments, and their morphological identification at the species level is traditionally difficult due to the well-known cryptic diversity and morphological plasticity typical of this genus, which have caused major taxonomic and nomenclatural confusion [58]. Several cryptic species within *Ulva* have been detected using genetic methods, such as DNA barcoding [59,60]. Unfortunately, for the purposes of this study it was not possible to obtain DNA sequence data from the *Ulva* samples analyzed; thus, future studies should investigate the taxonomical diversity of *Ulva* spp. in this area. To our knowledge, PUAs production, specifically of short- (i.e., C6:2 and C8:1) and medium-chain (i.e., C10:4) compounds by *S. muticum* is here reported for the first time (46.8 nmol/g), attesting the potential allelopathic effect of this species, which could contribute to its strong invasive potential.

The analysis of the microphytobenthic community revealed that diatoms were the dominant group, as typically reported, and as attested also in previous studies concerning the micro-epiphytic communities in this Adriatic area [24,29,61]. In these studies, Scanning Electron Microscopy (SEM) observations documented the great biodiversity of this community, while in the present study only light microscopy was used and only identifications at genera or even higher taxonomic levels could be obtained.

Microphytobenthic diatom populations are usually composed of adnate forms (strongly adhering horizontally to the substrate by means of the raphidic valve), which represent the first encrusting and more stable component in the diatom assemblage, or erect growth forms (adhering vertically to the substrate by means of mucous pillows or stalks/peduncles), which are less stable and colonize the substrate after the adnates, as well as motile forms (having high movement capability) [62]. Generally, the motile forms (e.g., *Nitzschia* and *Navicula* spp.) spread more effectively above the substrate than other forms but are less stable and can be easily removed by water movements. In this study, motile forms represented the main fraction in terms of cell abundance, probably due to the ability of these biraphid taxa to move within a mature substrate, which could make them superior competitors for nutrients and light [61]. Conversely, erect (e.g., *Grammatophora* and *Licmophora* spp.) or adnate (e.g., *Amphora* and *Cocconeis* spp.) forms reported low abundances (lower than 20%).

Notably, the microphytobenthic communities differed on thalli of the same macroalgae sampled in the two sites. In general, variations in the abundance of some diatoms (e.g., species belonging to the genera *Leptocylindrus* and *Cocconeis*, and unidentified pennate diatoms) were found at the Passetto. Conversely, in Porto variations were due to the abundance of motile forms (i.e., *Navicula* spp., *Pseudo-nitzschia* spp., and *Nitzschia* spp.),

which are able to move in response to a multitude of factors (e.g., light, hydrodynamics, tides, nutrient) and perhaps as a defence strategy against grazing or other stressors [63,64]. Similarly, a previous study [65] performed in the Adriatic Sea reported that the dominant taxa on the fronds of the invasive green alga *Caulerpa taxifolia* were characterized by high motility (e.g., *Navicula* and *Nitzschia* spp.), capable of moving on the substrate to find optimal conditions.

The results of this study highlighted differences in the taxonomic composition and abundance of epiphytic diatoms among the analyzed macroalgae and between the two sampling sites, as shown by MDS results where microphytobenthic communities clustered in two distinct groups. Differences among macroalgae could be explained, at least in part, by the different thallus architecture of the studied species, but also by other uninvestigated environmental factors reflecting site-specific conditions, which could be reflected in the structure and species composition of the algal communities. The population of the invasive alga *Sargassum muticum* in Porto may affect the community living under its canopy, especially considering its floating ability and long-branched habitat, which limits light penetration, together with other potential consequences caused by its presence (e.g., interactions with native species, competition for nutrients). In fact, benthic diatoms have a highly adaptive photosynthetic pigment apparatus, and can adapt well to low-light regimes [66]. In addition, different microalgal taxa have different light requirements and consequently light conditions can regulate colonization patterns of microalgal assemblages [67]. It should also be considered that the Porto site is within the area of a commercial harbor, and therefore represents a more stressful environment than the rocky coastal inlet of the Passetto site.

Concerning the meiobenthic community, harpacticoid copepods (including their *nauplii*) were the most abundant taxon, comprising 82.4% of the total meiofauna, followed by Gastropoda (7.8%) and juveniles of Amphipoda (2.2%). Nematodes accounted only for 1.9%, which only partially agrees with information reported in the literature [68]. Copepods have been reported as dominant in epiphytic environments with abundances ranging from 30 to 60% of the total meiofauna, followed by nematodes [69]. The ecological structure of the meiofauna community, in terms of relative abundance of major taxa, differed among macroalgae in the two sites. In Passetto, more diverse communities were found on all the four macroalgae, in particular on *D. polypoidioides* where the highest number of taxa was recorded and resulted more evenly distributed. On the contrary, in Porto the communities were mainly represented by copepod *nauplii* followed by harpacticoids. The average abundances of total meiofauna, however, did not show a clear pattern, with a general higher abundance on *D. dichotoma* and *D. polypoidioides* in Passetto and on *S. muticum* and *D. polypoidioides* in Porto. The community structure analyzed at major taxonomic levels resulted as significantly different among macroalgae and between sites on the same macroalga, except for communities associated to *Ulva* cf. *lacinulata*, which were not significantly different. Multivariate pairwise comparisons detected more significant differences among macroalgal species in the two sites than univariate post hoc. These different results between univariate and multivariate analyses suggested that differences among meiobenthic assemblages settled on macroalgae were mainly due to differences in the identity and relative abundance of meiofaunal taxa rather than to the total number of taxa and abundance [70]. These results agree with those reported by Richardson and Stephens [71], where *S. muticum* supported a lower diversity of meiofauna compared to native species, but disagree with Veiga et al. [14], who found that *S. muticum* apparently harbored more meiofaunal taxa than native macroalgae.

These contrasting results could be ascribed to differences in distribution of macroalgae between the two investigated sites. As also observed for microphytobenthic assemblages, in Passetto a more diversified macroalgal community was present, while in Porto *S. muticum* tended to dominate and grow until floating on the water surface, producing more sheltered and shaded conditions underneath. Therefore, we may speculate that different assemblages of macroalgal species may influence meiofauna communities [72]. In Passetto, a more diverse macroalgal community seems to offer a more stable habitat to meiofauna. Instead,

in Porto a less diverse macroalgal community dominated by *S. muticum* may explain the very high dominance of copepod *nauplii* and harpacticoids, that are well known for their high mobility [73].

Macroalgae, in addition to being key primary producers, provide the substrate for many organisms ranging from microbes to fish. Therefore, the community structure of a particular epibenthic community depends on the relationship of the different communities associated with different macroalgae. Many invertebrates use macroalgae as a refuge from physical stress, protection from predators, and many of them are herbivores that consume epiphytic algae or the host macrophyte itself [74]. Chemical defenses also play an important role in structuring associated epiphytic communities, as allelopathic compounds can reduce the settlement rate and development of sessile organisms. Furthermore, successful colonists must have a wide tolerance range and establish themselves during phases in which the composition and concentration of biomolecules are not harmful. Recently the importance of including the potential role of macroalgae, both in terms of structural complexity and production of allelochemical compounds, in the study of the interactions between different epibenthic associated organisms has been stressed [24].

Responses of microphytobenthic assemblages to PUAs production and macroalgal complexity, measured by fractal values (D), weight/area, volume/area, and perimeter/area ratios, showed that all the considered variables resulted significantly correlated with the differences in the microphytobenthos community structure. Thus, differences in microphytobenthos could be mainly due to variations in the morphological characteristics of the macroalgae but also to PUAs production by the different species.

On the contrary, responses of meiobenthic assemblages to macroalgal complexity measures and PUAs production showed that none of the considered variables were significant predictors of differences among the communities settled on macroalgae in the two sites. Many studies found that complexity did not correlate with invertebrate taxon richness, influencing only epifaunal abundance [75–77] or showing that complexity is not a consistent predictor of either the abundance or the diversity of the epifauna [78]. For example, Russo [70] and similarly Schreider et al. [79] showed that the complexity of algae did not explain amphipod abundance between macroalgal species with different structural complexity. Our results suggest that differences in the meiobenthic community structure may be mainly due to heterogeneity of macroalgal communities in terms of different species present in a site, while size and morphology of macroalgae have an unclear effect. The less structured meiobenthic community associated with the invasive species *S. muticum* seems to support this hypothesis.

Epiphytic communities could play a role in structuring meiobenthic associates assemblages [24]. In fact, when the DistLM analysis was performed, adding the total abundance of microphytobenthos, this variable resulted as a significant predictor of meiobenthic assemblage structure. This result agrees with Bologna and Heck [80] who showed a more important trophic role of epiphytes in structuring associated epibenthic assemblages than that of structural complexity, and with Lenzo et al. [24] who highlighted the role of microphytobenthos as an important driver in differentiating the meiofauna community among macroalgae. Indeed epiphytes could influence meiobenthic taxa by supplying food resource and adding complexity to the habitat [81]. The lack of correlation between PUAs and meiobenthic communities could be ascribed to the high taxonomic resolution used that might have failed to highlight the role of PUAs production on meiofauna, whose effects are known to be species-specific [24]. When the responses of meiofauna to PUAs and macroalgal complexity variables were analyzed including the number of dead copepods *nauplii*, dead harpacticoids, and the expelled egg sacs, whose release was reported as a way to reduce the load of toxic compounds [81], PUAs concentration became a significant predictor, suggesting that these compounds have some roles in structuring meiobenthic communities.

5. Conclusions

This study showed how two different macroalgal-associated communities (i.e., microphyto and meiobenthic assemblages) respond to the habitat provided by thalli of different species. Results showed that epibenthic communities respond differently to the variability of the macroalgal species and that, to better understand the global role of macroalgae as habitat formers on coastal ecosystems, it is necessary to analyze the interactions between organisms belonging to different trophic groups (e.g., microphytobenthos and meiofauna). The dominance of the large-sized invasive macroalga *Sargassum muticum* in Porto seemed to have a strong effect on the associated on the associated microphytobenthos, mainly characterized by motile diatoms (i.e., *Navicula* spp., *Pseudo-nitzschia* spp., and *Nitzschia* spp.) and meiofauna which was composed mainly by copepod *nauplii* and harpacticoids. The effects of invasive species on associated assemblages are difficult to predict and study as they depend on many factors, such as the identity of species and the macroalgal community structure, in terms of number and type of algae. Moreover, the interactions among complexity of macroalgae, the produced allelopathic compounds and the different epibenthic communities inhabiting them resulted very complex. The results of the present study highlight that when assessing the impact of the introduction of a non-native macroalga on the associated communities of a particular habitat and, more generally, on the environment, several aspects must be considered, including the allelochemicals potential of the macroalgae, which so far has been poorly considered. Further studies aimed at investigating the effects due to the invasiveness of macroalgal species should also consider the seasonal variability and should disentangle the effects of macroalgal complexity and allelochemical production on epibenthic communities performing field experiments using artificial macroalgae, which mimic substrates with different complexity, without being able to produce allelochemicals (e.g., PUAs).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w15091697/s1, Figure S1: Macroalgae sampled in Passetto (PAS) and Porto (POR): *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva* cf. *lacinulata* (UL), *Dictyopteris polypodioides* (DP) and *Sargassum muticum* (SM); Table S1: Results of PCA carried out on the variables measured for macroalgal morphology; Table S2: Results of PERMANOVA and pairwise comparison test carried out on morphological measure taken in Passetto (PAS) and Porto (POR) for each macroalga: *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva* cf. *lacinulata* (UL), *Dictyopteris polypodioides* (DP) and *Sargassum muticum* (SM); Table S3: Results of one-way PERMANOVA and pairwise comparisons carried out on the total concentration of PUAs taken in Passetto (PAS) and Porto (POR) for each macroalga: *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva* cf. *lacinulata* (UL), *Dictyopteris polypodioides* (DP) and *Sargassum muticum* (SM); Table S4: Microphythobenthos assemblages (cells/cm^2) associated to the macroalgae collected in Passetto (PAS) and Porto (POR): *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva* cf. *lacinulata* (UL), *Dictyopteris polypodioides* (DP) and *Sargassum muticum* (SM); Table S5: Results of one-way PERMANOVA and pairwise comparisons carried out on the number of taxa (S) and on total density (N) of microphytobenthos taken in Passetto (PAS) and Porto (POR) for each macroalga: *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva* cf. *lacinulata* (UL), *Dictyopteris polypodioides* (DP) and *Sargassum muticum* (SM); Table S6: Results of PERMANOVA and pairwise comparison test carried out on microphytobenthos community, taken in Passetto (PAS) and Porto (POR) for each macroalga: *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva* cf. *lacinulata* (UL), *Dictyopteris polypodioides* (DP) and *Sargassum muticum* (SM); Table S7: Results of SIMPER analysis based on four root transformed data, used to identify organisms that mostly contribute to microphytobenthos dissimilarity among site-macroalgae (Cut-off 60%). Av.Ab, mean abundance; Diss, mean dissimilarity; Diss/SD, dissimilarity/standard deviation; Contrib%, contribution relative to single taxon; Cum%, cumulative contribution; Table S8: Meiofauna assemblages (cells/cm^2) associated to the macroalgae collected in Passetto (PAS) and Porto (POR): *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva* cf. *lacinulata* (UL), *Dictyopteris polypodioides* (DP) and *Sargassum muticum* (SM); Table S9: Results of one-way PERMANOVA and pairwise comparisons carried out on the number of taxa (S) and on total density (N) of meiobenthos taken in Passetto (PAS) and Porto (POR) for each macroalga: *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva*

cf. *lacinulata* (UL), *Dictyopteris polypodioides* (DP) and *Sargassum muticum* (SM); Table S10: Results of PERMANOVA and pairwise comparison test carried out on meiobenthic community, taken in Passetto (PAS) and Porto (POR) for each macroalga: *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva* cf. *lacinulata* (UL), *Dictyopteris polypodioides* (DP) and *Sargassum muticum* (SM); Table S11: Results of SIMPER analysis based on four root transformed data, used to identify organisms that mostly contribute to meiobenthos dissimilarity among site-macroalgae (Cut-off 60%). Av.Ab, mean abundance; Diss, mean dissimilarity; Diss/SD, dissimilarity/standard deviation; Contrib%, contribution relative to single taxon; Cum%, cumulative contribution.

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