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METHOD

Ostreopsis cf. *ovata* abundances on different benthic substrata: how to compare them?

Stefano Accoroni[1,2] · Francesca Neri[1] · Marika Ubaldi[1] · Tiziana Romagnoli[1] · Cecilia Totti[1,3]

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Abstract
In the framework of benthic harmful algal bloom monitoring, the most common sampling strategy is based on the collection of macroalgae, and the abundance of epiphytic microalgae are mainly expressed as cells g^{-1} macroalgal fresh weight (fw). However, this methodology has some inherent problems, due to (i) the thallus-specific weights that markedly differ among algal species, (ii) the thallus architecture, and (iii) the production of allelopathic compounds that affects the epiphyte abundances among macroalgae, irrespective of the available colonizable surface. This study proposes a method to compare the abundances of *Ostreopsis* cf. *ovata* cells on different substrata, using a conversion factor that converts the abundances expressed as cells g^{-1} fw (or dry weight) to cells cm^{-2}. Expressing abundances in terms of cells cm^{-2}, the abundances can be compared (i) among different macroalgal species and (ii) between macroalgae and other substrata (such as rocks, pebbles, or shellfish shells). We also propose to normalize abundances when different macroalgae are sampled throughout the bloom period, considering the different epiphyte loads of different macroalgal species regardless of the available surface area.

Keywords Monitoring · Epiphytes · Harmful algae · Benthic dinoflagellates

Introduction

The dinoflagellate *Ostreopsis* cf. *ovata* is a benthic microalga able to produce toxins belonging to the palytoxin (PlTX) group (Nakajima et al. 1981; Meunier et al. 1997; Ciminiello et al. 2006; Yasumoto et al. 2007; Uchida et al. 2013; Brissard et al. 2015), recorded from tropical to temperate latitudes, with many records from Mediterranean coasts (Rhodes 2011; Parsons et al. 2012). Several environmental factors influence their abundance and bloom dynamics, including temperature, hydrodynamics, water depth, nutrient (both inorganic and organic) concentrations, substratum availability (Parsons and Preskitt 2007; Richlen and Lobel 2011; Glibert et al. 2012; Skinner et al. 2013; Accoroni et al. 2017a, b; Asnaghi et al. 2017; Pichierri et al. 2017; Boisnoir et al. 2018; Yong et al. 2018; Larsson et al. 2019; Pavaux et al. 2020). *Ostreopsis* cf. *ovata* grows in relatively shallow and well-illuminated waters attached to a variety of substrata, living either as epiphytic, epilithic, or epizoic. However, most studies have been conducted on macroalgae, where the abundance of benthic dinoflagellates is mostly quantified by collection of macroalgae which are shaken in ambient seawater to dislodge and suspend the attached cells. Then macroalgae are rinsed with filtered seawater (FSW) to optimize cell collection and thalli are weighted to determine their fresh weight (fw). Benthic dinoflagellate cells are generally enumerated using either a Utermöhl settling chamber (in case the sample needs to be concentrated in a sedimentation column) or a 1-ml Sedgewick Rafter chamber (Jauzein et al. 2018). Finally, cell abundances are expressed as a number of cells per gram of fresh weight of macroalgae (cells g^{-1} fw) (see Monserrat et al. 2022 for a review). The bloom trend is followed either on a single macroalgal species (e.g. Alkhatib et al. 2022; Ibghi et al. 2022) or on several ones, based on their availability in the sampling day/site (e.g.

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✉ Stefano Accoroni
s.accoroni@univpm.it

[1] Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, Via Brecce Bianche, 60131 Ancona, Italy

[2] Fano Marine Center, The Inter-Institute Center for Research on Marine Biodiversity, Resources and Biotechnologies (FMC), Viale Adriatico 1/N, 61032 Fano, Italy

[3] Consorzio Interuniversitario per le Scienze del Mare, CoNISMa, ULR Ancona, Ancona, Italy

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Mangialajo et al. 2008; Shears and Ross 2009; Gémin et al. 2020; Drouet et al. 2022).

Several studies underlined the importance of host thallus architecture (Lobel et al. 1988; Bomber et al. 1989). It has been observed that three-dimensional flexible thalli are more suitable for the accumulation of *Ostreopsis* spp. cells than other morphotypes, and branched thalli showed higher abundances than flattened thalli (mainly due to a different response of such thallus morphotypes to the wave action) (Vila et al. 2001; Totti et al. 2010; Cohu et al. 2013; Meroni et al. 2018). Moreover, given that *Ostreopsis* abundances are expressed as cells g^{-1} fw, abundance values are affected by the specific weight of the macroalgal substrata (Lobel et al. 1988; Parsons et al. 2017). This is very evident when comparing abundances between calcified and non-calcified macroalgae, or between small filamentous versus fleshy species. Normalization of abundance data to host surface area would be more informative and less subjected to misinterpretation than the more common units of cells g^{-1} (Lobel et al. 1988).

Interactions between *Ostreopsis* and macroalgal hosts are complicated by the presence of allelopathic compounds produced by the hosts. Macroalgae are known to produce a large number of secondary metabolites (Belghit et al. 2017; Pezzolesi et al. 2021). Several of these secondary metabolites can affect the growth, physiology, morphology, toxin production, and behavior of *Ostreopsis* cells, with a decrease in cell adherence, favoring or hampering the *Ostreopsis* colonization (Accoroni et al. 2015; Ben Gharbia et al. 2017; Ternon et al. 2020). Consequently, quantifying population trends of *Ostreopsis* at a location requires consistent collection of the same macroalgal substratum, as only data from the same host species would be comparable (Lobel et al. 1988). However, this approach is not always possible, as macroalgal species may disappear throughout the *Ostreopsis* bloom, due to several reasons, including their fast life cycle, making it necessary to sample other macroalgal species to follow the rest of the bloom. Moreover, given that each geographical area has its macroalgal community, it is often problematic to compare the *Ostreopsis* blooms around the world where macroalgal species composition markedly differs.

This study proposes a method to compare the abundances of *Ostreopsis* on different substrata using a conversion factor that converts, for each macroalgal species, the abundances expressed in cells g^{-1} fw to cells cm^{-2}. This method allows comparisons between abundances found on macroalgae having different thallus structure (e.g. fleshy vs calcareous, branched vs non-branched, etc.) and between macroalgae and other substrata (such as rocks, pebbles or shellfish shells) that would be otherwise incomparable. For this purpose, data gathered from the Conero Riviera (Ancona, northern Adriatic Sea, Italy) characterized by a rocky bottom were used, where blooms of *O.* cf. *ovata* have been constantly reported between the end of the summer and the beginning of the autumn since 2006 (Totti et al. 2010; Accoroni et al. 2011, 2012).

Finally, each macroalga has a different load capacity of epiphytic cells independently on the surface area, linked to both the complexity of the thallus (i.e. architecture, rugosity, presence of ephemeral structure such as hairs, and so on) and the allelochemical interactions. Therefore, this paper also proposes to normalize the epiphytic abundances between different macroalgal species, i.e. converting the abundances recorded on different macroalgae to obtain values as if they had always been recorded on the same macroalga (e.g. *Hypnea musciformis* in this study). In this way, it would be possible to follow the bloom trend also when different macroalgal species are sampled eliminating the bias related to changing algal substratum during the sampling period.

Materials and methods

Sampling and sample treatment

The study was carried out along the Conero Riviera (Ancona, northern Adriatic Sea, Italy) at the Passetto station (43°37′09″N, 13°31′54″E), a sheltered site affected by a moderate human impact, characterized by a rocky bottom and shallow depths.

Sampling of *Ostreopsis* cf. *ovata* was conducted weekly from July to November (i.e. covering the entire seasonal bloom period) from 2007 to 2014 on the following non-calcified macroalgae: *Ulva rigida* (non-branched, Ulvophyceae), *Hypnea musciformis* (branched, Rhodophyceae) and *Dictyota dichotoma* (non-branched), *Dictyopteris polypodioides* (branched) and *Gongolaria barbata* (formerly *Cystoseira barbata,* branched) (Phaeophyceae). Samples of macroalgal thalli (approximatively 10 g fw) were collected in three replicates (3 specimens for each species) at a depth of 0.5 m following the method described by Totti et al. (2010). Further macroalgae samples were collected to determine the fresh weight:area and dry weight:area ratios (see below).

In the laboratory, macroalgae were immediately treated following the method described by Totti et al. (2010). Samples of macroalgae were vigorously shaken in ~ 100 mL of seawater, in wide-necked HDPE sample bottles or plastic bags to dislodge the epiphytic cells. Thalli were then rinsed (three times) with FSW that was added to the storage water. Thalli were observed at the light microscope to check if the removal of epiphytic dinoflagellates was complete. Otherwise, further rinsing and shaking treatments were performed. The final water samples (only those for the *Ostreopsis* abundances estimation) were preserved with 0.8% neutralized formaldehyde (Throndsen 1978) and stored in the dark at 4 °C until microscope analysis. Then macroalgal

thalli were treated and weighted to determine fresh and dry weight (see below).

Determination of fresh and dry weight and assessment of the conversion factor (fresh weight:area and dry weight:area ratios) for each macroalgal species

Samples of macroalgal thalli of each species were first weighed to determine the fresh weight (g fw). The fresh weight was determined by weighing the thallus after dripping on absorbent paper to remove the external water. Then they were carefully placed on a photo scanner (EPSON Perfection V350 PHOTO, image resolution: 600 dpi) avoiding overlapping of thallus branches, and in case fragmented into smaller pieces (see Fig. 1 for an example of *Ulva rigida* thallus). The area (cm^2) of thallus fragments was calculated with image analysis software (Adobe Photoshop, Adobe Systems Incorporated, San José, CA, USA) on digitalized images obtained with the scanner. The scale tool of the image analysis software was set using an image of a ruler digitalized with the same scanner (measurement scale sets a specified number of pixels in the image equal to a number of scale units, such as centimeters). Then the area of each thallus fragment was measured with the measurement feature, selecting all the thallus images with the selection tool. The software calculated the area of the selected surface (i.e. the scanned surface of the thallus) expressed as cm^2. Considering that only one side of each macroalga was scanned, the thallus area was calculated by multiplying the scanned area by two. This approach has been adopted also for branched species having cylindric thalli, taking care to press the thallus on the scanner screen, as much as possible.

Fig. 1 Digitated image of *Ulva rigida*

Finally, the dry weight (dw) of the scanned macroalga was measured after it was kept in an oven (ISCO NSA90) for 48 h at 70 °C or 24 h at 104 °C.

The fw:area and the dw:area ratios were then calculated for each macroalgal species using replicate (from 11 to 22, see Table 1) to obtain a conversion factor, allowing the thallus area to be estimated only by measuring wet or dry weight.

Microscopy analysis of *Ostreopsis*

Ostreopsis abundances were estimated using an inverted microscope (Zeiss Axiovert 135) equipped with phase contrast, at 200 × magnification. Sub-samples (1–25 mL, depending on the *Ostreopsis* abundances) were settled in counting chambers after homogenization, according to the Utermöhl sedimentation method (Hasle 1978). The identification of *O.* cf. *ovata* was carried out by observing samples in epifluorescence after staining with a fluorochrome (Calcofluor White). Counting was performed on 10–30 random fields, 1–2 transects, or the whole sedimentation chamber, to count a representative cell number (at least 200 cells). Then the *Ostreopsis* cell abundances in the final water sample obtained from the macroalga treatment (see above) were

Table 1 Conversion factors obtained by the ratios (average ± standard deviation) of fresh weight to the area (fw:area) and dry weight to the area (dw:area) (g cm^{-2}) for *Hypnea musciformis, Dictyopteris polypodioides, Dictyota dichotoma, Gongolaria barbata*, and *Ulva rigida* thalli

	n	Fresh conversion factor (fw:area)	r^2 (fw-area)	Dry conversion factor (dw:area)	r^2 (dw-area)
Dictyopteris polypodioides	11	0.013406 ± 0.004122	***0.8127***	0.002295 ± 0.000581	***0.7932***
Dictyota dichotoma	22	0.013612 ± 0.006896	***0.8601***	0.001729 ± 0.000874	***0.9703***
Gongolaria barbata	18	0.003228 ± 0.002803	0.0285	0.000804 ± 0.000493	0.0116
Hypnea musciformis	11	0.006332 ± 0.001447	*0.5577*	0.000496 ± 0.000050	*0.6937*
Ulva rigida	22	0.011160 ± 0.006688	***0.5803***	0.001334 ± 0.000687	***0.8396***

Coefficient of determination (r^2) of Pearson correlation between fresh weight and area (fw-area), and dry weight and area (dw-area) for each macroalga are highlighted in italic when significant at $p < 0.05$, in bold italic when significant at $p < 0.01$, and in bold italic and underlined when significant at $p < 0.001$

calculated (cells mL^{-1}, Hasle 1978). Finally, the *Ostreopsis* abundances on macroalgae were expressed as cells g^{-1} fw, cells g^{-1} dw, and cells cm^{-2} of macroalga, as follows:

$$\text{cells g}^{-1}\text{ fw} = \frac{a \times V}{\text{fw}}$$

$$\text{cells g}^{-1}\text{ dw} = \frac{a \times V}{\text{dw}}$$

$$\text{cells cm}^{-2} = \frac{a \times V}{\text{area}}$$

where a is *Ostreopsis* abundance (cells mL^{-1}) in the final water sample, V is the volume of the final water sample (mL) of the treated macroalga, and fw, dw and area are fresh weight (g), dry weight (g) and area (cm^{-2}) of the thallus, respectively.

Normalization of *Ostreopsis* abundances between different algal species

Hypnea musciformis was the dominant and most sampled macroalgal species in our study area throughout the *Ostreopsis* cf. *ovata* blooms (Totti et al. 2010; Accoroni et al. 2022). For this reason, when *H. musciformis* occurred on the same sampling day with other macroalgal species, the ratio between (i) epiphyte abundances on *H. musciformis* and (ii) those on each other sampled species was calculated on a significant number of individuals. This ratio was adopted as the normalization factor allowing to convert the abundances on a given macroalga to those on *H. musciformis.* Normalization factor was calculated for both cells cm^{-2} and cells g^{-1} fw (given that most studies in the literature express epiphyte abundances as cells g^{-1} fw). Only *Ulva rigida* and *Dictyopteris polypodioides* were considered for the normalizing-factor calculation, while *Dictyota dichotoma* and *Gongolaria barbata* were excluded because they were rarely recorded on the same sampling day with *H. musciformis* (< 10 times).

To assess the usefulness of this approach, we first calculated the difference between the abundances (expressed as cells cm^{-2}) of *O.* cf. *ovata* recorded on *H. musciformis* and those on each other macroalgal species (*Ulva rigida* or *Dictyopteris polypodioides*) in each day where both species were sampled. Then, we calculated the same difference after normalizing (to *H. musciformis*) the abundance value of the other species.

Statistical analysis

The values of surface area of each sample of *Hypnea musciformis, Dictyopteris polypodioides, Dictyota dichotoma, Ulva rigida*, and *Gongolaria barbata* thalli were tested for significant correlation (Pearson) with its fresh and dry weight.

Results

Ostreopsis along the Conero Riviera

The *Ostreopsis*-abundance dataset is composed of 822 records distributed over 8 years of summer-fall *Ostreopsis* blooms. In this period, *Ulva rigida* and *Hypnea musciformis* were the macroalgal species mostly sampled (representing 31.0 and 26.3% of the entire dataset, respectively), followed by *Dictyopteris polypodioides, Dictyota dichotoma* and *Gongolaria barbata* (the 25.2, 13.1, and 4.4% of the entire dataset, respectively, Fig. 2).

The fresh weight:area and dry weight:area ratios

In total, 84 macroalgal thalli were analyzed to produce the conversion factor allowing the thallus area to be estimated

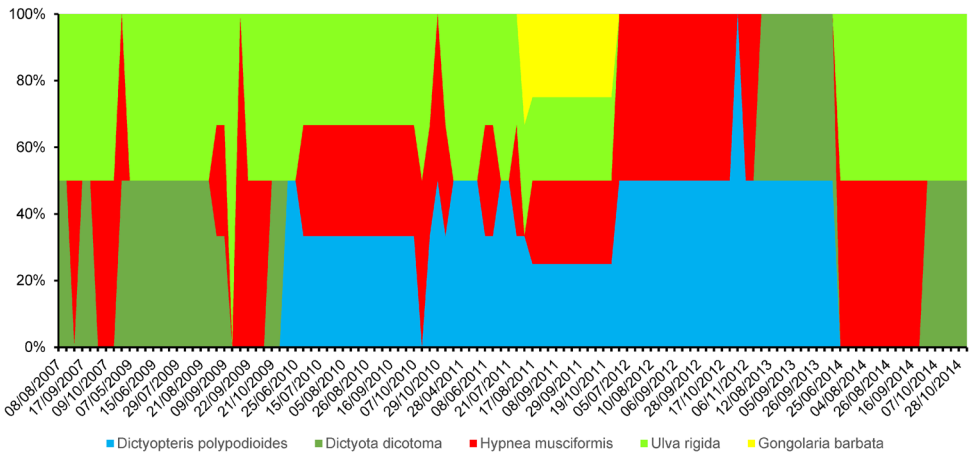

Fig. 2 Percent of macroalgal species sampled in the Passetto station during *Ostreopsis* cf. *ovata* blooms (from July to November 2007–2014): *Ulva rigida* (light green), *Hypnea musciformis* (red) *Dictyota dichotoma* (dark green), *Dictyopteris polypodioides* (light blue) and *Gongolaria barbata* (yellow)

only by measuring wet or dry weight. A significant positive correlation was observed between the values of thallus surface and those of thallus weight for all macroalgal species, except *Gongolaria barbata* (Table 1). The coefficient of determination (r^2) varied from 0.5577 to 0.8601 and from 0.6937 to 0.9703, for the fw-area and dw-area correlations, respectively.

The average value of the fw:area (or dw:area) ratio of a certain macroalga was the conversion factor (*cf*, g cm^{-2}) used to convert the weight (g) to surface (cm^{-2}), as follows:

$$\text{Thallus surface} = \frac{\text{fw}}{fcf} = \frac{\text{dw}}{dcf}$$

where fw and dw are the thallus fresh and dry weights, respectively, and *fcf* and *dcf* are the fresh and dry conversion factors of the given macroalgal species as indicated in Table 1.

Given the higher values of r^2 about the dw-area correlations than those about the fw-area correlations, the *dcf* was chosen as the best conversion factor to convert weight to surface in each macroalgal species.

Normalization of *Ostreopsis* abundances

Hypnea musciformis was the dominant macroalgal species in the study area throughout the *O.* cf. *ovata* blooms, therefore, abundances recorded on the same sampling day on different macroalgal species were normalized to those on *Hypnea musciformis*: the ratios between the abundances of *Ostreopsis* recorded on *Hypnea musciformis* (either cells g^{-1} fw or cells cm^{-2}), and those recorded on different macroalgal hosts in the same sampling day are reported in Table 2 and used as normalizing factor. The normalization to *Hypnea musciformis* was performed as follows:

$$[Hm] = [\text{sp.}] \times nf$$

where [*Hm*] is *Ostreopsis* abundance on *H. musciformis,* [sp.] is *Ostreopsis* abundance on a given macroalgal species and *nf* is its normalizing factor as indicated in Table 2.

The difference in abundances in *Hypnea musciformis* and *Ulva rigida* was considerably reduced after normalization (49,888 ± 34,019 and 17,466 ± 8,917 cells cm^{-2}, respectively), while the difference between abundances in *Hypnea musciformis* and *Dictyopteris polypodioides* was similar before and after normalization (1,769 ± 721 and 2,034 ± 661 cells cm^{-2}, respectively).

Discussion

Given the inherent problems linked to the monitoring of benthic harmful algal blooms performed mostly by the collection of macroalgae, some studies proposed some non-destructive alternative methods independent from macroalgae such as artificial substrata, e.g. nylon ropes (Faust 2009), PVC tiles (Parsons et al. 2017), pieces of fiberglass screens (Tester et al. 2014; Jauzein et al. 2016) or syringes (Abbate et al. 2012). However, the lack of a consistent correlation among epiphytic cell abundances on macroalgae versus artificial substrates was highlighted by Parsons et al. (2017), who suggested extreme caution when interpreting the data garnered from artificial substrate deployments. Moreover, the use of artificial substrata needs a period of incubation before collection. Recently, Mangialajo et al. (2017) have proposed a nondestructive quantification method for benthic dinoflagellate abundances called BEDI (Benthic Dinoflagellates Integrator), where benthic dinoflagellates are resuspended within a hollow plastic cylinder for quantifying abundances as cells per unit of seabed surface area. Anyway, most studies have been conducted on macroalgae by expressing epiphyte abundances as cells g^{-1} fw. Moreover, given that collecting macroalgae is a time-consuming and destructive approach, the Environmental Agencies perform the monitoring only on the water column and, therefore, guidelines for the management of *Ostreopsis* blooms in Mediterranean countries adopted threshold levels expressed as cells L^{-1} (Asnaghi et al. 2017).

In this work, we recommend a method that allows to compare abundances from different macroalgal species in surface units of macroalgae. This approach solves the problem of the different specific weight of distinct macroalgae, that affects the abundance values when expressed as cells g^{-1} fw or dw. The significant fw-area and dw-area correlations demonstrated that weight data (both wet and dry) could be reasonably converted to algal surface area

Table 2 Normalizing factors obtained by the ratio (average ± standard deviation) of *Ostreopsis* abundances (cells g^{-1} fw and cells cm^{-2}) on *Hypnea musciformis* and other co-occurring macroalgal species: *Hypnea musciformis-Ulva rigida* and *Hypnea musciformis-Dictyopteris polypodioides*

	n	Normalizing factors on *Hypnea musciformis* (cells g^{-1} fw)	Normalizing factors on *Hypnea musciformis* (cells cm^{-2})
Ulva rigida	36	16.8851 ± 29.0840	33.7141 ± 40.4615
Dictyopteris polypodioides	33	2.8327 ± 3.6654	2.0180 ± 1.7229

for the species examined, except for *Gongolaria barbata*, probably due to the morphological and seasonal variation of fronds: *Gongolaria barbata* has a complex thallus composed by phylloids, branchlets and stipe, only the last of which is perennial (Falace and Bressan 2006). This last observation suggests that this approach is not applicable to all macroalgal species (especially those having complex morphotypes where specific weight differs among thallus parts). Beyond *Hypnea musciformis, Dictyopteris polypodioides, Dictyota dichotoma* and *Ulva rigida* from this study, this approach was suitable also for *Dictyota cervicornis*, *Halimeda incrassata*, *Laurencia gemmifera* and *Laurencia intricata* in the Florida Keys (Parsons et al. 2017). Moreover, looking at the r^2 values, it is evident that the best way to convert weight to surface in each macroalgal species was from the dry weight. Since the fresh weight is determined by weighing the thallus after the removal of excess water by adsorbent paper, this variable may be substantially affected by operator handling and environmental conditions (e.g. measurement performed on a hot dry day or a wet cold day). Conversely, dry weight is less affected by operator handling because all the water (both intracellular and extracellular) is removed by oven drying. Thus, expressing the abundances in surface unit (cells cm^{-2}) allows comparisons between the abundances found on different substrata. For example, thanks to this approach it was possible (i) to appreciate the differences between the abundances of *Ostreopsis* on macroalgae with different degrees of thallus complexity, demonstrating that branched macroalgae showed significantly higher abundances than non-branched one (Totti et al. 2010), and (ii) to compare the *Ostreopsis* abundances on macroalgae and hard substrata (rocks, pebbles or mussel shells), highlighting that hard substrata supported significantly higher abundances than macroalgal thalli (Totti et al. 2010; Accoroni et al. 2011).

Furthermore, by normalizing the abundances (i.e. converting the abundances recorded on different macroalgae to obtain values as if they had always been recorded on the same macroalga), we can appreciate the bloom trend even by sampling different species throughout the sampling period. The simple conversion from grams to the surface area would not be sufficient for this aim, because each macroalga, independently from the available colonizable surface, could favor or discourage the growth of *Ostreopsis*. This could be due to both (i) the complexity of the thallus (e.g. the branched thalli favor higher abundances, Totti et al. 2010) and (ii) the presence of allelopathic substances, which can vary during the bloom period, and can discourage or favor the *Ostreopsis* growth (Ternon et al. 2020). In this study, the abundances recorded on all the sampled macroalgae were normalized to those on *Hypnea musciformis* as it was the most available macroalgal species in the study area during the *Ostreopsis* blooms. After normalization, the differences in *Ostreopsis* abundance between *Hypnea musciformis* and *Ulva rigida* considerably decreased, highlighting the usefulness of this approach. On the contrary, the normalization of *Dictyopteris polypodioides* did not affect such difference, resulting in less usefulness. These results are not surprising considering that the thallus morphology of *Hypnea musciformis* and *Dictyopteris polypodioides* are both branched while that of *Ulva rigida* is flat.

For this reason, the usefulness of normalizing the abundance values is clearer when macroalgae having different morphotypes are considered.

In conclusion, it is obvious that the best way to follow a *Ostreopsis* bloom remains to sampling always the same macroalgal host because it is not possible to appreciate the limits of this normalization. Indeed this normalization is an approximation that is assumed to be linear, although there could be a species-specific plateau of abundances per algal surface unit. Moreover, it does not take into consideration that thallus complexity (including thallus structure, rugosity, presence of ephemeral structure such as hairs, etc.), thallus-specific weight, and allelochemical production may vary throughout the life and annual cycles of the macroalgae and/or depending on environmental factors of the site. However, if it is not possible to sample the same macroalgal species (or substrata where abundances evaluation can be directly performed on surface unit, such as pebbles, rocks or artificial substrata), this approach represents a good approximation and could be adopted for estimating microepiphytes (including benthic diatoms) colonization on any macroalgal communities all around the world.

Author contributions All authors contributed to the study's conception and design. Material preparation and data collection were performed by SA. Data analysis, synthesis and drafts of the manuscripts were written and edited by all the authors. All the authors approved the final version of the manuscript.

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Data availability Datasets generated during the current study are not publicly available but are available from the corresponding author on request.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Ethical approval All applicable international and institutional guidelines for sampling, care and experimental use of animals for the study have been followed.

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