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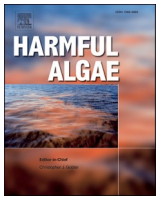

Basin scale variability of *Ostreopsis* spp. blooms provides evidence of effectiveness of an integrated sampling approach

Mariachiara Chiantore [a,b,c], Valentina Asnaghi [a,b,c,*], Marie Abboud-Abi Saab [d], Laury Acaf [d,e], Stefano Accoroni [b,f], Ali Badreddine [d], Laura Escalera [g,h], Anna Fricke [i,j], Cécile Jauzein [e,k], Rodolphe Lemée [e], Cecilia Totti [b,f], Souad Turki [l], Magda Vila [m], Imen Zaghmourii [l], Adriana Zingone [g], Elisa Berdalet [m,1], Luisa Mangialajo [i,1]

[a] DiSTAV, Università di Genova, C. so Europa 26, 16132 Genoa, Italy
[b] CoNISMa, Piazzale Flaminio 9, 00196 Rome, Italy
[c] National Biodiversity Future Center, 90133 Palermo, Italy
[d] National Council for Scientific Research, National Centre for Marine Sciences, P.O. Box 534, Batroun, Lebanon
[e] Sorbonne Université, CNRS, Laboratoire d'Océanographie de Villefranche, 06234 Villefranche-sur-mer, France
[f] Department of Life and Environmental Sciences, Università Politecnica delle Marche, via Brecce Bianche, 60131 Ancona, Italy
[g] Stazione Zoologica Anton Dohrn, Napoli, Italy
[h] Subida a Radiofaro 50, 36390 Vigo (Pontevedra, Spain), Centro Oceanografico de Vigo (IEO-CSIC), Spain
[i] Université Côte d'Azur, CNRS, ECOMERS, Parc Valrose 28, Avenue Valrose, 06108 Nice, France
[j] IGZ - Leibniz Institute of Vegetable and Ornamental Crops, e.V. Theodor-Echtermeyer-Weg 1, 14979 Großbeeren, Germany
[k] Laboratoire d'Ecologie Pélagique (PDG-ODE-DYNECO-PELAGOS) Centre Bretagne - ZI de la Pointe du Diable - CS 10070 - 29280 Plouzané, France
[l] National Institute of Marine Sciences and Technologies, 28 rue 2 mars 1934, Carthage Salammbô, Tunisia
[m] Institut de Ciències del Mar (CSIC), Passeig Marítim de la Barceloneta, 37-49, 08003 Barcelona, Catalonia, Spain

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Dr. C. Gobler

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ABSTRACT

Ostreopsis spp. blooms have been occurring in the last two decades in the Mediterranean Sea in association with a variety of biotic and abiotic substrata (macroalgae, seagrasses, benthic invertebrates, sand, pebbles and rocks). Cells proliferate attached to the surfaces through mucilaginous trichocysts, which lump together microalgal cells, and can also be found in the plankton and on floating aggregates: such tychoplanktonic behavior makes the quantitative assessment of blooms more difficult than planktonic or benthic ones. Different techniques have been so far applied for quantifying cell abundances of benthic microalgae for research, monitoring and risk assessment purposes. In this context, the Benthic Dinoflagellates Integrator (BEDI), a non-destructive quantification method for benthic dinoflagellate abundances, was developed and tested within the EU ENPI-CBCMED project M3-HABs. This device allows mechanical detachment of cells without collecting the benthic substrate, providing an integrated assessment of both epiphytic and planktonic cells, i.e. of the number of cells potentially made available in the water volume from "resuspension" which could have harmful effects on other organisms (including humans).

The present study confirms the effectiveness of the BEDI sampling device across different environments across the Mediterranean Sea and constitutes the first large-scale study of *Ostreopsis* spp. blooms magnitude in function of different macro- and meso-habitat features across the basin.

1. Introduction

Benthic Harmful Algal Blooms (B-HABs) are an increasingly common phenomenon at temperate and subtropical latitudes that may represent a serious threat to other organisms (including humans) and the environment. B-HABs started receiving larger interest since the end of the XXth century, because certain harmful benthic species have been identified and/or caused blooms in temperate areas, gaining the attention of both the scientific community and public governance. Benthic dinoflagellates thrive in shallow and well illuminated waters, growing

* Corresponding author.
E-mail address: valentina.asnaghi@unige.it (V. Asnaghi).
[1] The last two authors equally contributed and share last name on the manuscript.

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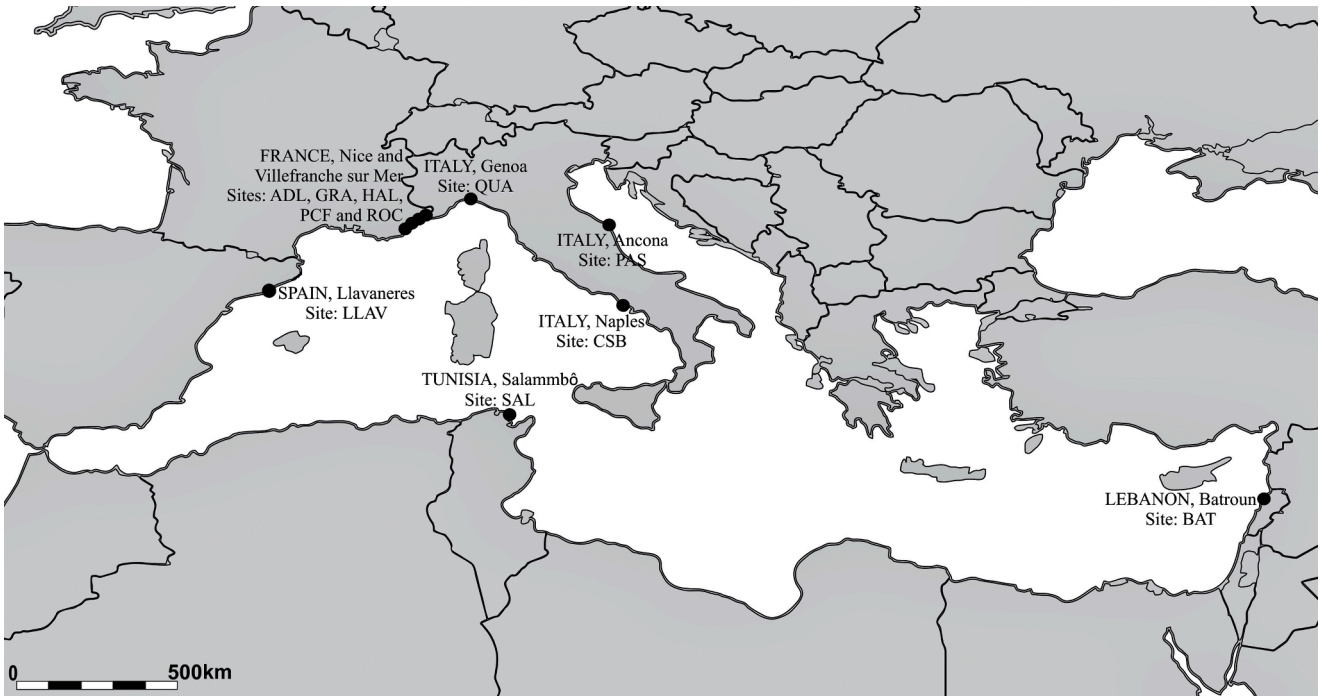

Fig. 1. Location of sampling sites along the Mediterranean coast.

on several substrates (e.g., macrophytes, rocks, corals, mollusk shells, sand), where they are attached by the sticky mucilage they produce, exhibiting host-macrophyte preferences (see Parsons and Preskitt, 2007 and references therein; Rains and Parsons, 2015; Boisnoir et al., 2019). These microalgae can detach from the benthos by potential internal rhythms and actively move in the surrounding water or can also be easily released by mechanical action (e.g., waves, anchoring, trampling), becoming part of the plankton (Pavaux et al. 2021). In these situations, they form floating aggregates at the sea surface (see e.g., Mangialajo et al., 2017) that become visible when high cell abundances are reached.

The degree of attachment of the different species may differ (Giussani et al., 2017; Boisnoir et al., 2019) and consequently the relative ratio of released versus attached cells densities, i.e. of planktonic versus benthic populations, may vary among species and under different environmental conditions. Such tychoplanktonic behavior makes the quantitative assessment of blooms more difficult than to proper planktonic or benthic species. For this reason, different techniques have been so far applied for quantifying cell abundances of benthic microalgae for research, monitoring and risk assessment purposes (Berdalet et al., 2012; Tester et al., 2014; Giussani et al., 2017; Mangialajo et al., 2017; Jauzein et al., 2018).

Among B-HABs taxa, *Ostreopsis* spp. have been recorded in many locations along the Mediterranean (Accoroni et al., 2016), Australian (Verma et al., 2016), New Zealand (Shears and Ross, 2009) and Japanese coasts (Parsons et al., 2012). Specifically, in the Mediterranean Sea, two genotypes corresponding to the morphotypes *O.* cf. *ovata* Fukuyo and *O.* cf. *siamensis* Johs.Schmidt were recorded (Penna et al. 2010, 2012), and more recently a new species from the eastern area (Cyprus and Lebanon) was described, *O. fattorussoi* Accoroni, Romagnoli & Totti (Accoroni et al., 2016). In line with the wide geographical distribution of this genus, *Ostreopsis* spp. are found in different seascapes where they express their proliferation capability. Almost all knowledge about the ecology of *Ostreopsis* spp. in the Mediterranean Sea mainly refers to *O.* cf. *ovata* due to its dominance over the other species (*O.* cf. *siamensis* and *O. fattorussoi*) in this area (Battocchi et al., 2010; Accoroni et al., 2016).

In the Mediterranean Sea, *Ostreopsis* spp. live associated with a variety of biotic and abiotic substrata (macroalgae, seagrasses, benthic invertebrates, sand, pebbles and rocks; Totti et al., 2010; Meroni et al., 2018; Monserrat et al., 2022), attached through mucilaginous trichocysts, which lump together microalgal cells (Honsell et al., 2013; Escalera et al., 2014).

In general, *O.* cf. *ovata* proliferates during the summer season, from June to September/October, within the 22 °C to 24 °C sea temperature range, especially in relatively sheltered and shallow-water areas (Mangialajo et al., 2008; Pezzolesi et al., 2012; Vila et al., 2016; Asnaghi et al., 2017; Drouet et al., 2022). In the NW Adriatic Sea, the maximum abundance is usually reached in October when temperatures start to decrease (Accoroni et al., 2012). During long lasting blooms, its mucilaginous matrix can form a brownish mat that may be detached (*sensu* Moreira et al., 2016) by mechanical action or local hydrodynamics. In consequence, the concentration of *O.* cf. *ovata* in the water is generally related to its abundance in the benthic habitat which is considered the population stock or reservoir (Mangialajo et al., 2011). Thus, for the appropriate monitoring of the blooms, sampling both plankton and benthos is usually advised.

The two most commonly applied approaches for *Ostreopsis* spp. abundance assessment are the quantification on benthic substrates (epiphytic, measured as cells per gram of sampled substrate, mostly macroalgae or cells per mm^2 of rocks (e.g. Accoroni et al., 2011) and in the water in the proximity of macroalgal communities or abiotic substratum (planktonic, measured as cells per liter). Collecting samples for estimating planktonic cells density is easy but environmental conditions (e.g., waves, trampling, daily migration of cells) result in a great variability at a small spatial and temporal scale and a poor relationship with the benthic cells density. Additionally, a long processing time is required for planktonic cells abundance estimation, since due to the relatively low levels, the cells are left overnight or for 48 h to settle in a 50 mL or 100 mL Utermöhl chamber before being counted in the microscope. Recently, automated cells counting software have been developed (Vassalli et al., 2018) at least to reduce the identification and counting phase, although not solving the long settling time yet.

Measures of benthic cell density may also be variable in relation with the substrate (i.e., as a function of the specific weight and shape of the sampled macroalga or other *substratum*; Meroni et al., 2018; Monserrat et al., 2022) and small scale variability of the rocky shores and, usually,

Table 1
Sampling sites (Country, Site, Acronym, Coordinates, Region) and periods.

Country	Site	Site acronym	Coordinates (Lat N; Long E)	Region	2015 Sampling Period	2016 Sampling Period	2017 Sampling Period
France	Anse des Fosses	ADF	43° 41′ 11″; 7°20′ 15″	Ligurian – Lig_Sea	25 Jun - 13 Aug		
France	Grasseuil	GRA	43°41′59″; 07°19′20″	Ligurian – Lig_Sea	2 Jul - 6 Oct	21 Jun-23 Aug	
France	Haliotis	HAL	34°40′38″; 07°13′51″	Ligurian – Lig_Sea	18 Jun - 13 Aug	21 Jun-23 Aug	
France	Port Cap Ferrat	PCF	43° 41′ 2444; 7° 20′ 11″	Ligurian – Lig_Sea	2 Jul - 13 Aug		
France	Rochambeau	ROC	43°41′35″; 07°18′31″	Ligurian – Lig_Sea	26 Jun - 13 Aug	21 Jun-23 Aug	
Italy	Genova - Quarto dei Mille	QUA	44°23′17″; 08°59′37″	Ligurian – Lig_Sea	30 Jun - 10 Sept	21 Jun-4 Nov	21 Jun-21 Aug
Italy	Naples - Cala San Basilio	CSB	40°47′34″; 14°11′16″	Tyrrhenian – Tyrr_Sea	15 Jul-27 Jul		
Italy	Ancona - Passetto	PAS	43°37′09″; 13°31′53″	Adriatic – Adr_Sea	5 Aug-9 Nov		
Lebanon	Batroun	BAT	34°06′52″; 35°38′54″	East Med	17 Jul–23 Oct		
Spain	Llavaneres	LLAV	41°33′ 7.73″; 2°29′ 31.77″	West Med – W_Med	14 Jul-15 Sept		
Tunisia	Salammbô	SAL	36°50′34″; 10°19′37″	East/South Med – E_S_Med	18 Aug-8 Sept		

Table 2
Summary of physical and biological features of each sampling site as described in detail in the "Sampling sites" section. Exposure to prevalent waves (EX: Exposed; SH: Sheltered), Dominant macroalgal species sampled, Habitat Complexity Category (H: High; M: Medium), Slope (G: Gentle; S: Steep), Seascape at 50 m radius (Rock_N: 100 % natural rock; Rock_A: 100 % artificial rock; Rock_Peb: patches of natural rocky bottom interspersed in a pebble bottom; Rock_N_Seagr: seascape dominated by patches of natural rocky bottom interspersed in a seagrass meadow), Geomorphology (distance of the 10 m depth contour, in meters, from the coastline) of the coast at the sampling sites.

Site	Exposure	Dominant macroalgal species	Habitat Complexity Category	Slope of the sea bottom	Seascape	Geomorphology (m)
ADF	SH	*Halopteris/Cystoseira sensu lato/Dictyota/Jania*	H	G	Rock_N_Seagr	350
GRA	SH/EX	*Dictyota/Cystoseira s.l.*	H	G	Rock_N	320
HAL	EX	*Halopteris/Dictyota*	M	S	Rock_A	60
PCF	EX	Sphacelariales (*Sphacelaria*+*Halopteris*)/*Jania*	M	S	Rock_A	100
ROC	SH	*Halopteris/Dictyota*	M	S	Rock_N	70
QUA	SH/EX	*Halopteris*	M	G	Rock_N	264
CSB	SH	*Asparagopsis/Dictyota*	M	G	Rock_Peb	200
PAS	SH	*Dictyota/Chondria/Ulva*	H	G	Rock_Peb	143
BAT	SH/EX	*Ellisolandia elongata*	M	S	Rock_N	40
LLAV	EX	*Jania*	M	G	Rock_N	1000
SAL	SH/EX	*Enteromorpha/Sargassum/Ulva*	M	S	Rock_N_Seagr	170

do not allow the direct comparison among blooms over large spatial and temporal scales (Mangialajo et al., 2017). However, it is generally agreed that the quantification of epiphytic cells is more accurate than the concentrations of cells in the plankton, and that it better represents the potential risk associated with the bloom. Yet, the quantification of cells in the benthos requires the collection of macroalgae or rocks and it is, unfortunately, a destructive method that should be avoided in large scale and long-term monitoring, especially on canopy forming species (or corals in tropical habitats).

In this context, the Benthic Dinoflagellates Integrator (BEDI), a non-destructive quantification method for benthic dinoflagellate abundances, was developed and tested within the EU ENPI-CBCMED project M3-HABs (Mangialajo et al., 2017). The rationale behind the BEDI assessment method is that mechanical detachment of cells enables the quantification of abundances as cells per unit of seabed surface area (i.e., cells·mm^{-2}) or as "Potentially Resuspended" cells per unit of volume (PRcells·ml^{-1}), by integrating both epiphytic and planktonic cells, therefore providing an estimate of the number of cells potentially made available in the water volume from detachment or "resuspension" (as defined in Mangialajo et al., 2017). This method has presently been applied on a relatively small spatial scale for *Ostreopsis* cf. *ovata* bloom monitoring (Mangialajo et al., 2017) and research (Accoroni et al. 2017), encompassing only a small set of different seascapes. Yet, since the BEDI cell abundance assessment is less dependent on the *substratum* than other methods (*i.e.* macroalgal species or rocks) or the dominant ecosystem (i.e., algal forests or turfs, seagrass beds, coral reefs), it represents a potentially robust sampling method allowing the comparison of benthic dinoflagellate blooms over broad spatial scales, thus allowing for habitat scale bloom assessments.

Another non-destructive method allowing the quantification of benthic dinoflagellates is the deployment of artificial substrates (screen sampling method, *sensu* Tester et al., 2014) widely applied in tropical areas (Tester et al., 2014; Parson et al., 2017; Fernandez-Zabala et al., 2019; Fernandez-Zabala et al., 2022; Tester et al., 2022). This method has also been used for *Ostreopsis* spp. in the Mediterranean Sea in the context of M3-HABs (see Jauzein et al., 2016), but it has the drawback that is needed to sample twice to get the result.

The present study shows the results of the application of the BEDI sampling device for testing its effectiveness across a quite large range of conditions. It also constitutes the first large-scale study of *Ostreopsis* spp. blooms magnitude in function of different macro- and meso-habitat features across the Mediterranean basin.

2. Material and methods

2.1. Sampling sites

Samples to estimate *Ostreopsis* spp. abundances were collected at 11 Mediterranean sites (Fig. 1; Table 1) in the coasts of Lebanon, Tunisia, Spain, France and Italy. Samples were obtained during summer 2015, except for the Ligurian Sea, where samplings were also conducted in 2016 and 2017 in QUA (Italy) and in 2016 in ROC, GRA and HAL (France). We refer to *Ostreopsis* spp. since the dominant species in the area is *O.* cf *ovata* (Casabianca et al., 2014), but in the Lebanon region *O. fattorussoi* is the dominant species (Accoroni et al., 2016), while *O. siamensis* although present in different locations it reaches low concentrations (Mangialajo et al., 2017).

In each sampling site, dominant macroalgal species were recorded and a Habitat Complexity Category (HCC) was attributed according to the dominant macroalgae (H: High, canopy forming macroalgae >10

Table 3
List of the macroalgal species collected at the sampling sites. See Table 1 for sampling site acronyms.

Taxa	Acronym	Sample sites (number of samples)
Acetabularia acetabulum (Linnaeus) P.C. Silva	ACE	GRA (4) ROC (2)
Asparagopsis taxiformis (Delile) Trevisan de Saint-Léon	ASP	CSB (9)
Chondria sp.	CHO	PAS (43)
Dictyota sp.	DIC	GRA (5) HAL (13) ROC (17)
Ellisolandia elongata (J.Ellis & Solander) K. R. Hind & G.W. Saunders	ELL	BAT (27) HAL (2)
Halopteris scoparia (Linnaeus) Sauvageau	HAL	ADF (10) GRA (17) HAL (30) PCF (6) QUA (105) ROC (20)
Hypnea musciformis (Wulfen) J.V. Lamouroux	HYP	PAS (8)
Jania rubens (Linnaeus) J.V. Lamouroux	JAN	GRA (10) HAL (8) LLAV (11) PCF (4) ROC (11)
Laurencia complex	LAU	GRA (1) HAL (3) PAS (2) PCF (1)
Padina pavonica (Linnaeus) Thivy	PAD	ADF (8) GRA (11) HAL (2) PCF (3) ROC (6)
Ulvales	ULV	SAL (5)

cm, i.e. Fucales; M: Medium, erect macroalgae around 2–10 cm height, e.g., *Halopteris, Dictyota, Padina*; L: Low, turf forming macroalgae, shorter than 2 cm height; Table 2). Medium HCC was dominant in all sites together with patches of High complexity categories in some sites.

Sites were characterized in terms of Exposure, based on LEK – Local Expertise Knowledge (EX: Exposed, if facing directly prevalent winds; SH: Sheltered, in case of protected conditions to main winds and waves), Slope at the sampling site (G: Gentle, sub-horizontal, $0° < x < 30°$; S: Steep, $30° < x < 60°$), Seascape at 50 m radius (Rock_N: 100 % natural rock; Rock_A: 100 % artificial rock; Rock_Peb: patches of natural rocky bottom interspersed in a pebble bottom; Rock_N_Seagr: seascape dominated by patches of natural rocky bottom interspersed in a seagrass meadow) and Geomorphology of the coast (distance of the 10 m depth contour, in meters, from the coastline: < 100 m and >100 m; Table 2).

2.2. Sampling

Plankton samples (PLK, 250–1000 mL) were collected at 20 cm above the substrate (corresponding to around 0.3 m depth). Dominant macroalgae in each sampling site (Table 3) were collected at 0.5 m depth, using the protocol reported in Mangialajo et al. (2011) and Jauzein et al. (2018) and treated for the quantification of epiphytic cells (EPI).

BEDI samples were collected at the same bottom depth of the EPI samples, using the device described in Mangialajo et al. (2017). It consists of a hollow aluminum cylinder, open at both ends, with a height of 70 cm and a diameter of 25 cm, corresponding to a sampled area of 491 cm^2. A rubber seal is fixed to the bottom of the device, in order to avoid cell loss when sampling irregular seabed. During BEDI sampling, the device is placed over the seabed and the water inside is vigorously stirred using a standard hand-paddle, allowing the detachment of cells from the biofilm and their homogenization with the cells in the surrounding water. The water must be mixed for a few seconds (usually 5 s), in order to allow complete detachment of cells and mixing of the water, avoiding the loss of cells from the bottom. Immediately after stirring, a sample is taken from the center of the BEDI column using a 250 mL plastic bottle.

For each site, samples were collected with the following sequence: surrounding water (PLK), macroalgae (EPI) and BEDI, in order to avoid a mechanical detachment of epiphytic cells that might artificially increase PLK cell concentrations.

The temporal sampling was adapted to cover the bloom phase of *Ostreopsis* in each surveyed Mediterranean region. In the northern Mediterranean Sea, blooms have often occurred between July and August, extending also in September–October. Accordingly, monitoring of such blooms was performed from June to October, with a sampling frequency of at least once per week (up to 2–3 days per week) during the bloom period.

2.3. Sample processing and cell counting

All samples (PLK, EPI and BEDI) were fixed with 1 % acidic Lugol solution and stored at +4 °C in the dark until microscopy counting analyses.

For PLK samples, the Utermöhl method was used (Utermöhl, 1958), where a sub-sample (50 mL in the standardized protocol for *Ostreopsis*

Table 4
Concentrations of *Ostreopsis* spp. cells (minimum, maximum and average values) in the plankton (PLK), as epiphytes (EPI) and collected by the BEDI device in the different sampling sites and periods in 2015, 2016 and 2017.

	Number of samples	Sampling period	*PLK* cells·L^{-1} min-max	*PLK* cells·L^{-1} avg	*EPI* cells·gFW^{-1} min-max	*EPI* cells·gFW^{-1} avg	*BEDI* cells·mm^{-2} min-max	*BEDI* cells·mm^{-2} avg	*BEDI* PRcells·L^{-1} min-max	*BEDI* PRcells·L^{-1} avg
2015										
France	108	Jun-Oct	0 - 37,080	2111	0 - 1663,011	10,395	0 - 514	35	0 - 1028,000	77,000
Italy	110	Jun_Nov	0 - 147,736	5287	0 - 2947,842	270,139	0 - 1316	117	0 - 3400,000	298,000
Lebanon	27	Jul_Oct	0 - 1120	182	114 - 11,453	3232	0 - 2	1	0 – 6000	2000
Spain	14	Jul_October	13 - 39,690	11,850	13,573 - 3397,215	572,817	1 - 2169	513	3000 - 4820,000	1416,000
Tunisia	5	Aug_Sept	1080 - 54,920	18,168	13,012 - 106,089	59,137	11 - 31	22	198,000 – 494,000	358,000
2016										
France	89	Jun_Aug	0 - 157,000	12,370	0 - 2357,982	217,075	0 - 717	105	1000 - 1668,000	208,000
Italy	42	Jun_Nov	0 - 26,080	3201	1929 - 4119,695	410,161	2 - 2091	194	5000 - 5973,000	532,000
2017										
Italy	16	Jun_Aug	60 - 81,380	14,304	6559 - 2890,528	806,844	1 - 1266	260	2000 - 4220,000	802,000

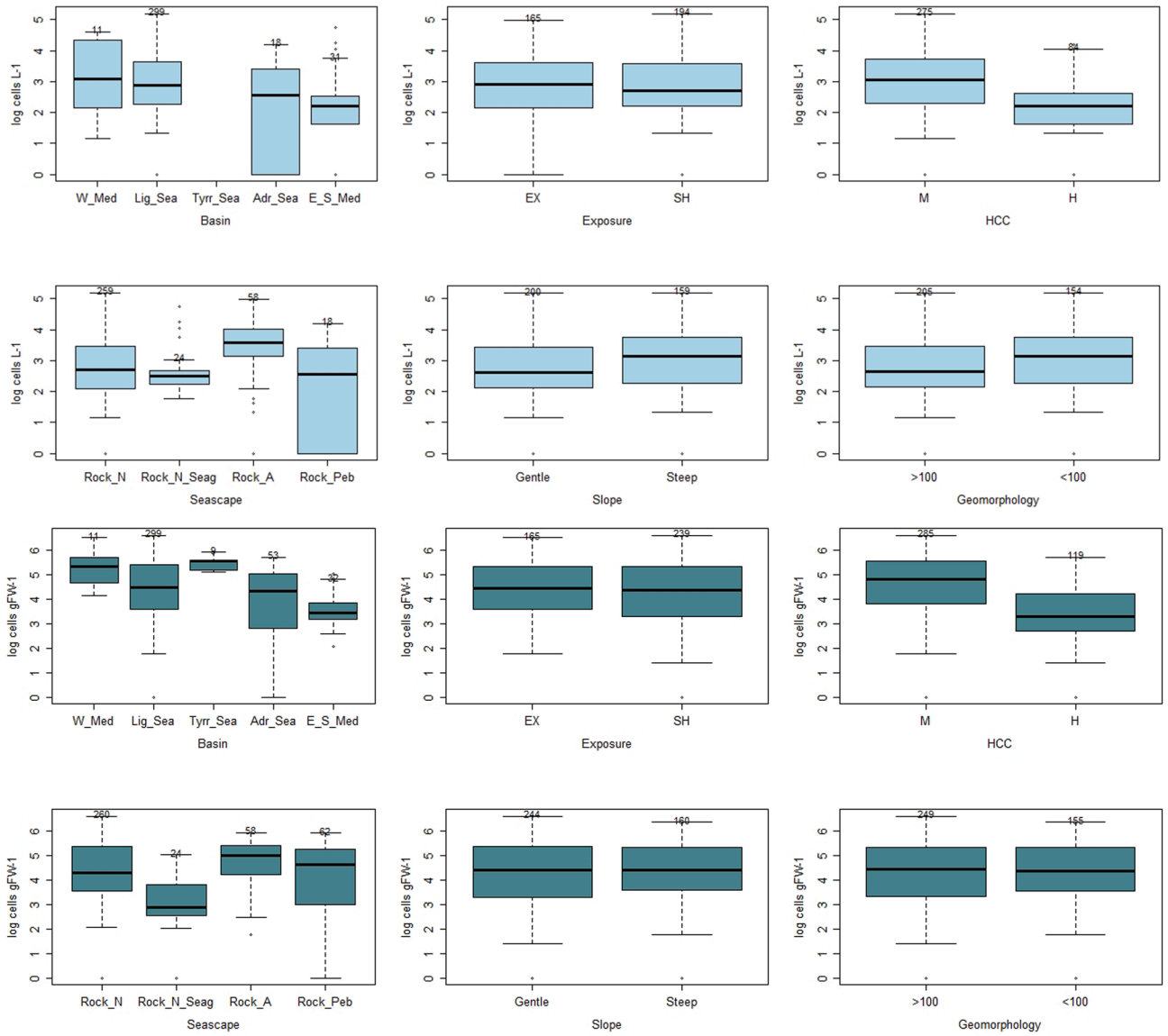

Fig. 2. a: Comparison of *Ostreopsis* spp. cell abundance in the plankton according to Basin, Exposure, Habitat Complexity Category, Seascape, Slope and Geomorphology. Medians are highlighted in bold; bars represent the 25 % and 75 % quartiles; whiskers represent the lowest and highest data points, asterisks are outliers. Numbers on top of each box represent the number of observations.
Fig. 2b: Comparison of *Ostreopsis* spp. cell abundance as epiphytic cells on macroalgae according to Basin, Exposure, Habitat Complexity Category, Seascape, Slope and Geomorphology. Medians are highlighted in bold; bars represent the 25 % and 75 % quartiles; whiskers represent the lowest and highest data points, asterisks are outliers. Numbers on top of each box represent the number of observations.
Fig. 2c: Comparison of *Ostreopsis* spp. cell abundance in the BEDI samples, according to, Exposure, Habitat Complexity Category, Seascape, Slope and Geomorphology. Medians are highlighted in bold; bars represent the 25 % and 75 % quartiles; whiskers represent the lowest and highest data points, asterisks are outliers. Numbers on top of each box represent the number of observations.

spp; Jauzein et al., 2018) was poured in a cylinder/chamber complex and allowed to settle for at least 24 h before observation at the inverted microscope. Counting was performed on the whole surface of the chamber or, in case of high concentration, along two orthogonal transects (cross-like).

Concerning EPI samples, the separation of cells from macroalgae was performed by shaking the samples vigorously for 10 s, then rinsing and shaking again the macroalgae twice with filtered seawater (0.2–0.45 μm). Counting was performed on 1 to 10 mL of this rinsing water containing released *Ostreopsis* benthic cells, using the Sedgewick Rafter or Utermöhl chambers. The number of PLK cells was subtracted from the cells in the rinsing water in order to account for the EPI cells only. The macroalgae were then rinsed and weighted to assess their fresh weight and report the number of EPI cells as cells·gFW^{-1}.

BEDI samples do not need any particular treatment and cells were quantified on 1 mL sea water, using a Sedgewick Rafter chamber. At least, 200 cells were counted, when possible, in the whole chamber or in parts of the chamber.

Cell abundances are reported as cells per unit of volume (cells·L^{-1}) for PLK samples, cells per unit of Fresh Weight of macroalga (cells·gFW^{-1}) for EPI samples, and as cells per unit of sea-bottom surface (cells·mm^{-2}) or as Potentially Resuspended cells per unit of volume (PRcells·L^{-1}) for BEDI ones, which represents the concentration in the water column potentially caused by detachment of EPI cells.

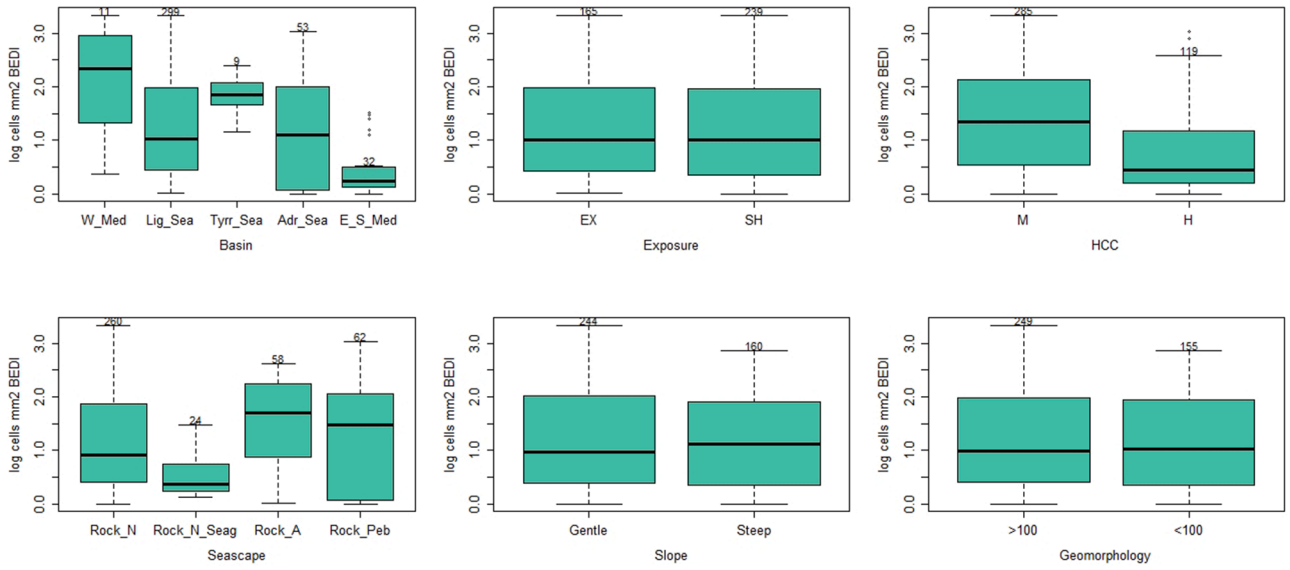

Fig. 2. (*continued*).

Table 5
Results of Kruskall-Wallis test performed on PLK (cells·L^{-1}), EPI (cells·gFW^{-1}) and BEDI (cells·mm^{-2}) samples, testing for pairwise differences (by Nemenyi test) among levels of the different factors (Basin, Exposure, Habitat Complexity, Seascape, Slope, Geomorphology and Sampled Species). Post-hoc significance codes: *** p-value<0.001; ** p-value<0.01; * p-value<0.05. Lig_Sea: Ligurian Sea; E-S Med: East-South Mediterranean; W Med: Western Mediterranean; Tyrr Sea: Tyrrhenian Sea; Adr Sea: Adriatic Sea. See Table 3 for macroalgae acronym concerning the Sampled species factors.

	PLK (cells·L^{-1})	EPI (cells·gFW^{-1})	BEDI (cells·mm^{-2})
Basin	11.6, p-value = **0.009**	46.9, p-value < **0.0001**	37.5, p-value < **0.0001**
Post-hoc	(Lig_Sea = *W* Med) > *E*-S Med*	(Lig Sea = Tyrr Sea = *W* Med) > *E*-S Med***; Tyrr Sea > Adr Sea**; W Med > Adr Sea*	(Lig Sea = Tyrr Sea = *W* Med) > *E*-S Med***; W Med > Adr Sea *
Exposure	0.48, p-value=0.49	0.48, p-value=0.49	0.18, p-value= 0.67
Habitat Complexity	25.6, p-value < **0.0001**	86.4, p-value < **0.0001**	43.6, p-value < **0.0001**
Post-hoc	(*H* < *M*)	(*H* < *M*)	(*H* < *M*)
Seascape	31.2, p-value < **0.0001**	26.8, p-value < **0.0001**	21.2, p-value < **0.0001**
Post-hoc	(Rock_N_Seagr = Rock_N) < Rock_A***	(Rock _*A* = Rock_*N* = Rock_Peb) > Rock_N_Seagr ***	(Rock_*A* = Rock_*N* = Rock_Peb) > Rock_N_Seagr ***
Slope	12.08, p-value= **0.0005**	0.42, p-value=0.52	0.04, p-value=0.83
Geomorphology	8.4, p-value=**0.004**	0.29, p-value=0.59	0.11, p-value=0.74
Sampled species (see table 3)		90.6, p-value < **0.0001**	77.1, p-value < **0.0001**
Post-hoc		(ELL = PAD = HYP) < (HAL = ROCK = DIC = ASP)**	(ELL = PAD = HYP) < (HAL = ROCK = DIC = ASP = CHO = JAN) **

2.4. Data analysis

Data were $\log_{10}$ transformed to be displayed and analyzed. Boxplots of cell abundances (for PLK, EPI and BEDI data) were produced according to the following factors: Basin, Exposure, Habitat Complexity Category, Seascape, Slope and Geomorphology. EPI data were also plotted according to the macroalgal species sampled.

Scatterplots to investigate fitting of EPI and BEDI data were performed according to the different investigated factors, with the complementary factor Site. Spearman non-parametric coefficient (rho) was calculated to assess the correlation between the two variables (given their non-normal distribution).

Additionally, to investigate the consistency of EPI and BEDI correlation across spatial and temporal scales, scatterplots and Spearman coefficients have been plotted separately for each Basin and for the three different sampled years in the QUA site.

Similarly, a scatterplot for log PLK cells (cells·L^{-1}) and log Potentially Resuspended cells (PRcells·L^{-1}), for PLK cell concentrations over 100 cells·L^{-1}, was performed and the Spearman coefficient calculated.

Given the non-normal distribution of response variables, non-parametric Kruskall-Wallis analysis of variance was performed on cell abundances (for PLK, EPI and BEDI data) to test the effect of the different factors and of the sampled species. Nemenyi *post-hoc* tests were performed on significant differences.

To assess the role of the investigated factors on bloom occurrence, a Random Forest Model was built (Breiman, 2001) on $\log_{10}$ transformed BEDI data (cells·mm^{-2}), using as predictors the Sampled Species, Sampling Year, Sampling Month and Sampling Day in addition to the six above-considered factors. The Random Forest Model (Breiman, 2001) grows an ensemble of trees using independent observations. For each tree, only a sub-sample ("out-of-bag") of the data is used for the training. Besides, only a random subset of predictor variables is considered for split point selection at each node. Each tree was grown using a bootstrapped sample containing about 60 % of all the data, with 5 features tried at each split. A total of 5000 trees were grown and feature importance was assessed as mean decrease in accuracy, expressed as mean square error (MSE), when out-of-bag data for that variable were permuted while all others were left unchanged. Goodness of fit for this model was visually assessed by plotting average predicted values versus logged observations. After this preliminary visual assessment, the R^2 statistic was computed for a numerical evaluation of the correlation between predicted and observed values.

All statistical analyses were performed using the software R (R Core Team, 2015).

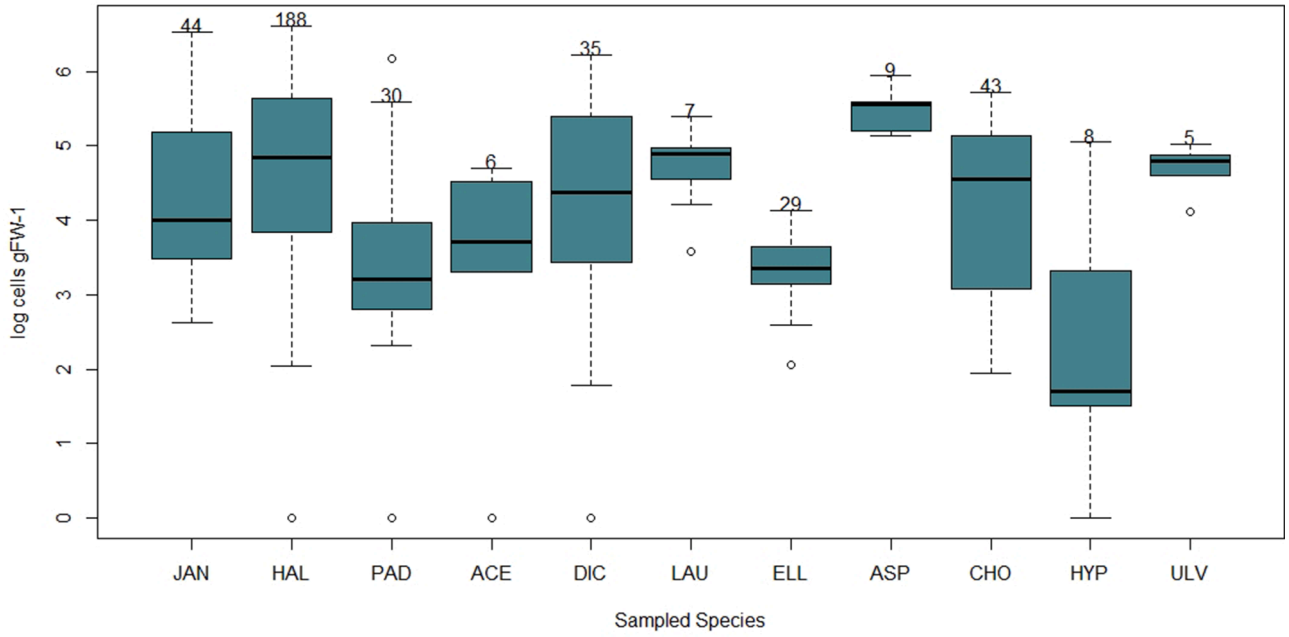

Fig. 3. Boxplots reporting $\log_{10}$ abundance of *Ostreopsis* spp. cells·gFW^{-1} in EPI samples, according to sampled macroalgal species (see Table 3 for abbreviations). Medians are highlighted in bold; bars represent the 25 % and 75 % quartiles; whiskers represent the lowest and highest data points, asterisks are outliers. The number of samples per boxplot is indicated at the top of each one.

3. Results

3.1. Regional and seascape variability of Ostreopsis spp. blooms

A total of 408 samples were collected across the study area and sampling years for EPI and BEDI and a total of 362 for PLK. Ranges and averages of PLK (cells·L^{-1}), EPI (cells·gFW^{-1}) and BEDI (cells·mm^{-2} and PRcells·L^{-1}) concentrations are reported in Table 4.

The highest cell abundance in the plankton (157,000 cells·L^{-1}) was recorded in France, at the ROC site, on 19/07/2016, whereas the highest average value over the sampling period was recorded in Tunisia at the SAL site (18,168 cells·L^{-1}), between 18/08/2015 and 08/09/2015.

The highest abundance of epiphytic cells was recorded in Italy, in the QUA site, on 26/07/2016, reaching 4119,695 cells·gFW^{-1}. In the same site, also the highest average value over the sampling period was recorded (806,844 cells·gFW^{-1}) between 21/06/2017 and 21/08/2017.

The highest abundance of integrated cells collected with BEDI device was recorded in Spain, in the LLAV site on 14/07/2015, reaching 2169 cells·mm^{-2}. In the same site, also the highest average value over the sampling period was recorded (513 cells·mm^{-2}), between 14/07/2015 and 15/09/2015. In terms of potentially resuspended cells (PR BEDI data), the largest value was recorded in Italy (QUA), on 22/07/2016, reaching 5973,000 PRcells·L^{-1}, while the average highest value (1416,000 PRcells·L^{-1}) was recorded in Spain between 14/07/2015 and 15/09/2015 (LLAV).

Log_{10} concentrations of PLK cells·L^{-1}, of EPI cells gFW^{-1} and of BEDI cells mm^{-2} across Basins, Exposures, Habitat Complexity Categories, Seascapes, Slopes and Geomorphologies are plotted in Fig. 2a, 2b and 2c, respectively.

All investigated variables (*i.e.*, the abundances of PLK (cells·L^{-1}), EPI (cells·gFW^{-1}) and BEDI samples (cells·mm^{-2}) displayed a large variability across Basins (Table 5): in all cases, the south-eastern Mediterranean basin exhibited lower median values. No differences across Exposure conditions were observed for any of the variables, both for cells adhered to the substrates (EPI and BEDI samples) and for suspended cells (PLK samples). Slope and Geomorphology showed significant effects only on PLK samples, with higher median values in a generally steeper condition (when Slope = Steep and Geomorphology = <100 m). Stronger differences were detected across Habitat Complexity Categories, being H condition lower in cell abundances compared to the M one, particularly in EPI and BEDI samples, and across Seascapes, with lower cell abundances in the natural rocks interspersed with seagrass compared to all other categories.

Concerning the relationships between individual macroalgal species and *Ostreopsis* abundances (Fig. 3), a large scatter was detected for the macroalgal species collected in a larger number of samples, therefore through a larger spatial and/or temporal range. *Ellisolandia elongata, Hypnea musciformis* and *Padina pavonica* displayed lower concentrations in both the EPI samples and the integrated BEDI samples (Table 5).

3.2. BEDI performance across regional and seascape variability

The abundances of integrated cells measured using the BEDI device fitted quite well with the epiphytic abundances (Spearman coefficient rho = 0.90; p value < 0.001), across the different sites and investigated factors (Fig. 4), confirming that the abundances of integrated cells measured using the BEDI device represent a good proxy of the concentration of the benthic stock, across the different sites and investigated factors.

The consistency of the relationship between EPI and BEDI estimates across space and time is shown in Fig. 5 and 6 respectively. The Spearman correlation coefficient between $\log_{10}$ BEDI cells (cells·mm^{-2}) and $\log_{10}$ EPI cell abundance (cells·gFW^{-1}) for each Basin showed similar values: rho showed high correlation values for all the considered Basins, ranging from 0.77 to 0.89 (Fig. 5).

The temporal scale tested in Quarto dei Mille (QUA) is completely superimposable across the three different sampled years, with almost equal Spearman rho values ranging between 0.85 and 0.93 (Fig. 6).

A good, even if lower, correlation (Spearman coefficient= 0.79; p value < 0.001) was found between potentially resuspended cells (PRcells·L^{-1}), measured through BEDI, and the number of plankton cells in seawater (PLK; Fig. 7), taking into account only data >100 cells·L^{-1}.

The results from the Random Forest Model provide a good percentage of variance explained (R^2 = 0.72; Fig. 8a), between predicted and observed $\log_{10}$ transformed BEDI data (cells·mm^{-2}). Most significant

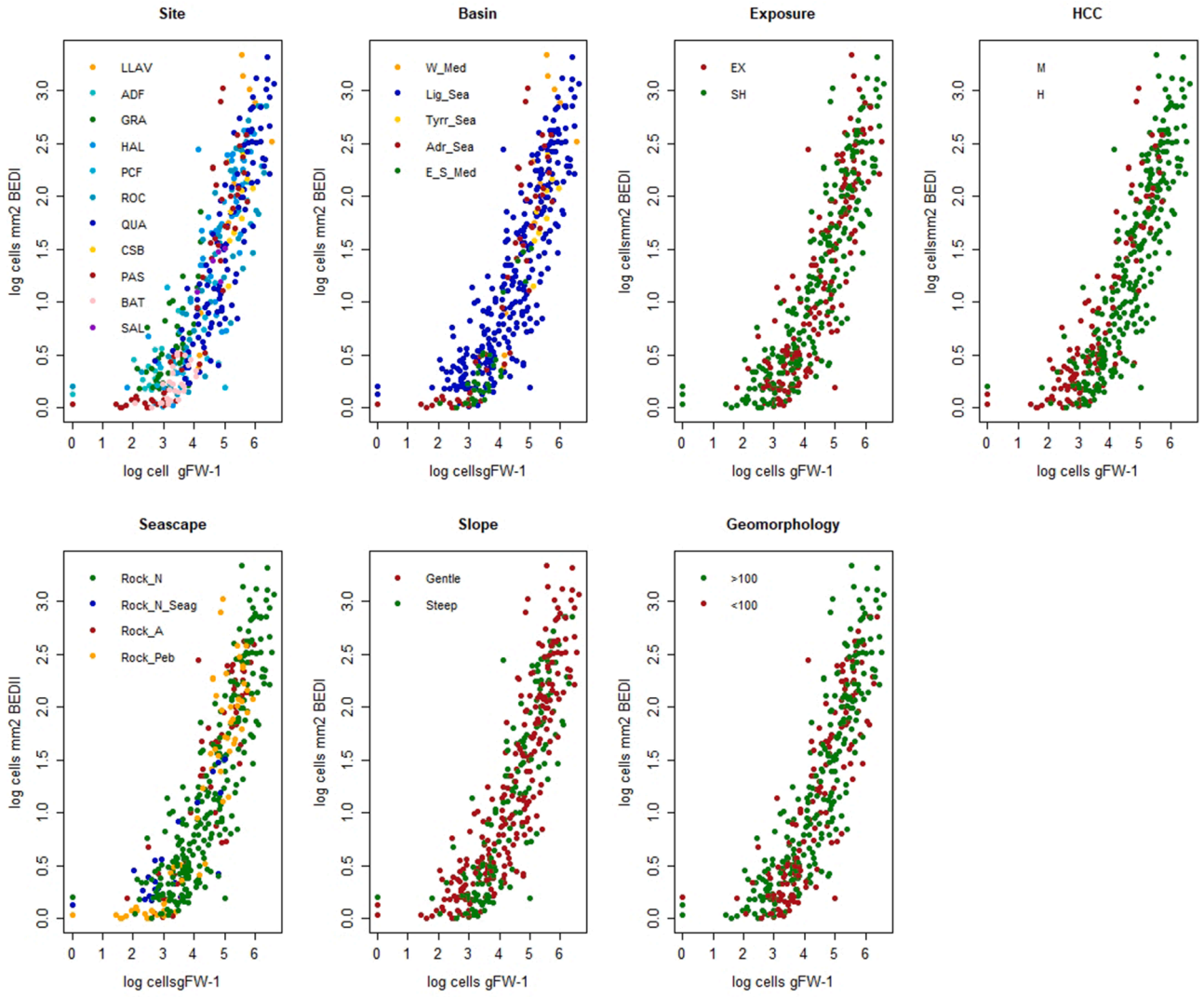

Fig. 4. Correlation between $\log_{10}$ EPI cell abundance (cells·gFW^{-1}) and $\log_{10}$ BEDI cells (cells·mm^{-2}), displayed according to Site (SAL, Tunisia; BAT, Lebanon; HAL, GRA, ROC, ADF, PCF, France; QUA, Liguria, Italy; PAS, Marche, Italy; CSB, Campania, Italy; LLAV, Spain), Basin (E_S_Med: East-South Med; W_Med: West-Med; Lig_Sea: Ligurian Sea; Tyrr_Sea: Tyrrhenian Sea; Adr_Sea: Adriatic Sea), Exposure (NE: not exposed; E: exposed), Habitat Complexity (M: medium; H: high), Seascape (Rock_N; Rock_N_Seagr; Rock_Peb; Rock_A), Slope (Gentle; Steep), Geomorphology (<100: distance to 10 m depth contour <100 m; >100: distance to 10 m depth contour >100 m).

variables were Habitat Complexity Category and Month, followed by sampled species (Fig. 8b).

4. Discussion and conclusions

Ostreopsis spp. blooms in the study area exhibited a large range of variability across the investigated regions. As far as the Basin scale comparison, differences could be related to the environmental variability of key factors (such as temperature and salinity, e.g., Accoroni and Totti, 2016 and references therein; Asnaghi et al. 2017) that could differently promote bloom magnitude as well as to the possible occurrence of different species (such as *O. fattorussoi*, found so far in the Eastern Mediterranean; Accoroni et al. 2016; Açaf et al., 2020). Additionally, the large variability in bloom intensity at the basin scale, in terms of PLK, EPI and BEDI cells concentrations could depend on the different number of samples (Table 4) and the temporal duration of the sampling season in the different sites (Table 1).

Differences in the concentration of cells in the plankton (PLK) resulted to be correlated with large scale factors, such as Basin (higher concentrations in the Ligurian Sea/West Mediterranean), Seascape (higher values on artificial rock substrates, compared to natural rocky seascapes), Slope (higher values along steeper shores) and Geomorphology (higher values in areas where the 10 m isobath is closer than 100 m from the shore), while not correlated to Exposure of the site. Significant differences in PLK cells concentrations were also found according to Habitat Complexity Category: the High level (dominance of macroalgal species longer than 10 cm) provided lower values than the Medium one.

As far as cells present on the substrates (EPI and BEDI cells), differences in concentrations were found across the Mediterranean Sea basins (with higher values in the Western Mediterranean compared to the Adriatic and South-East Mediterranean), while no significant differences were detected across Exposure, Slopes and Geomorphology levels. Conversely, significant differences were detected across Seascapes, Habitat Complexity Categories and Sampled species, providing evidence of how local factors (both abiotic and biotic) are stronger for epiphytic cells than broad scale and topographic variables. In terms of Seascape, lower values were recorded on mixed natural rocky shore/seagrass meadows compared to natural and artificial rocky shores and pebbles. As far as Habitat Complexity Category, the Medium level (erect

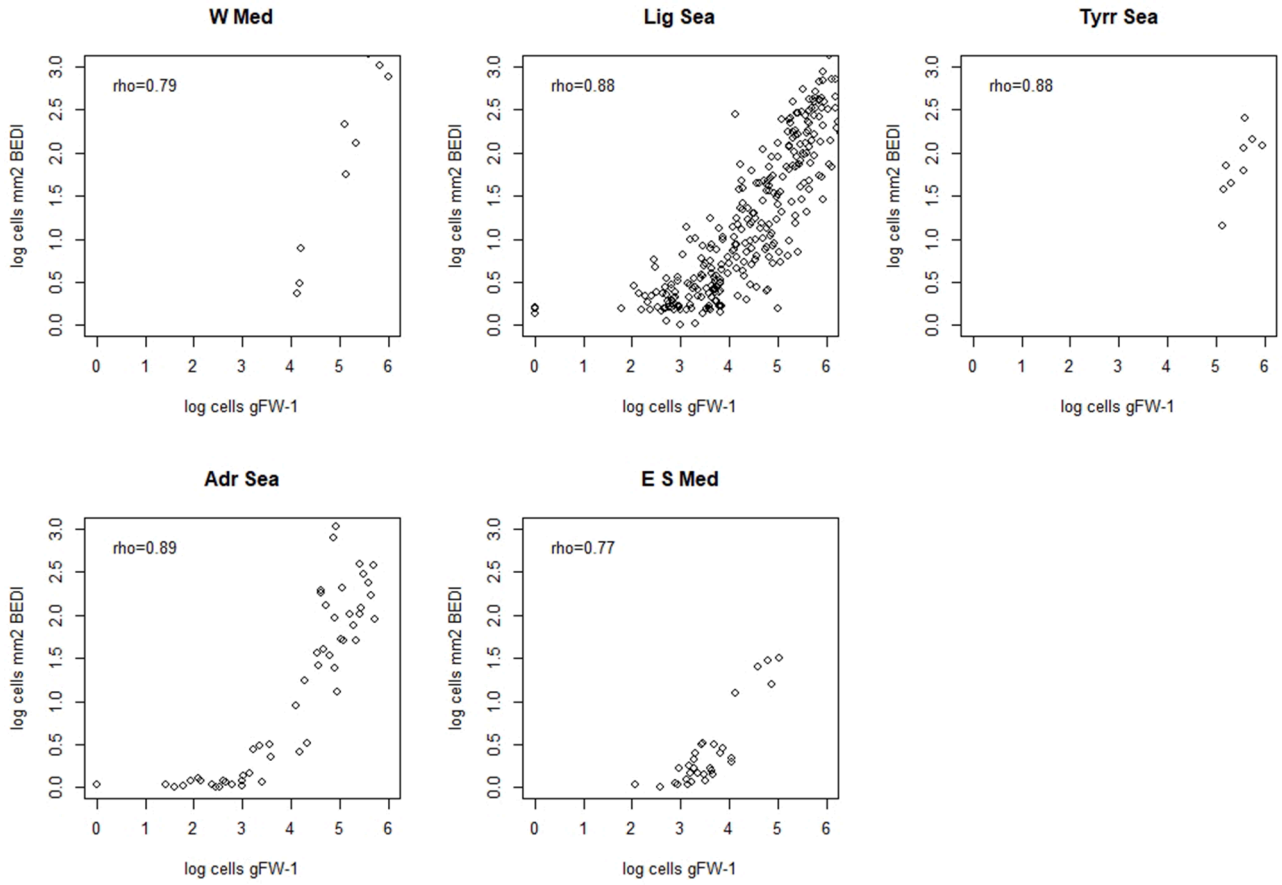

Fig. 5. Spearman correlation between $\log_{10}$ BEDI cells (cells·mm^{-2}) and $\log_{10}$ EPI cell abundance (cells·gFW^{-1}) for each Basin.

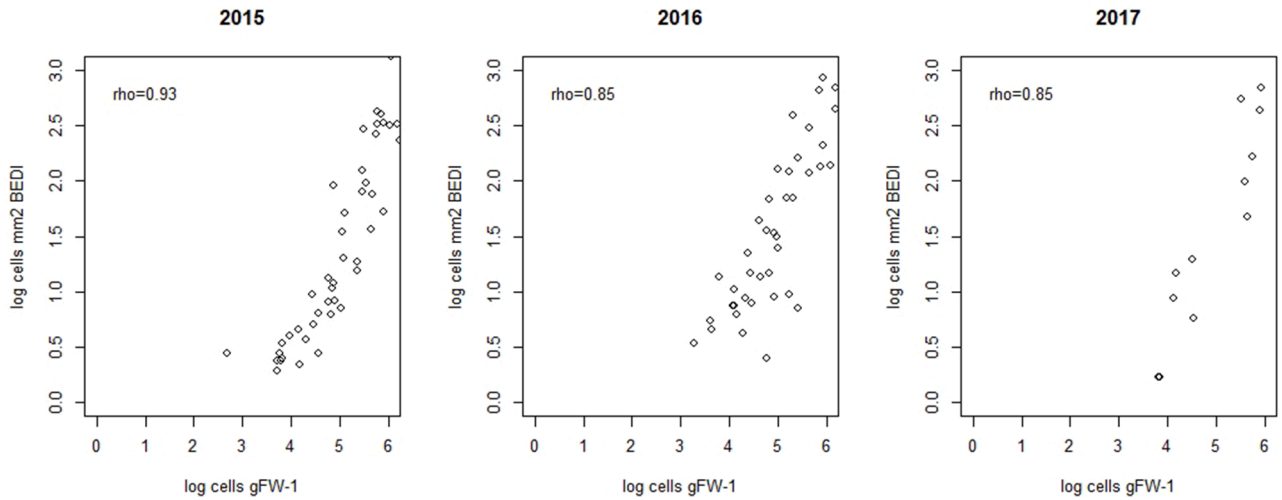

Fig. 6. Spearman correlation between $\log_{10}$ BEDI cells (cells·mm^{-2}) and $\log_{10}$ EPI cell abundance (cells·gFW^{-1}) across time in Quarto dei Mille (QUA) – Ligurian Sea.

macroalgae from 2 to 10 cm height) displayed higher concentrations of cells, compared to the High level (mostly canopy forming dominated assemblages). At the macroalgal species level, *Ellisolandia elongata, Hypnea musciformis* and *Padina pavonica* displayed lower concentrations in both the EPI samples and the integrated BEDI samples. EPI samples were not collected for the canopy-forming species in order not to affect the assemblage. In this sense, as BEDI is not destructive, is an excellent sampling system for high level complexity habitats.

Exposure at the small scale (the one addressed in the present study) is not a key indicator of bloom suitability for any of the variables investigated (PLK, EPI and BEDI), while Habitat Complexity Category may be a good indicator of bloom suitability, both in terms of macrophyte community complexity and type: high complexity communities, such as canopy-forming dominated assemblages and seagrass meadows display a lower abundance of *Ostreopsis* cells compared to medium complexity seaweeds (i.e., erect), independently of the specific geographic area, as noted by earlier studies, and discussed below).

Slope and Geomorphology seem to affect in particular PLK cells, but

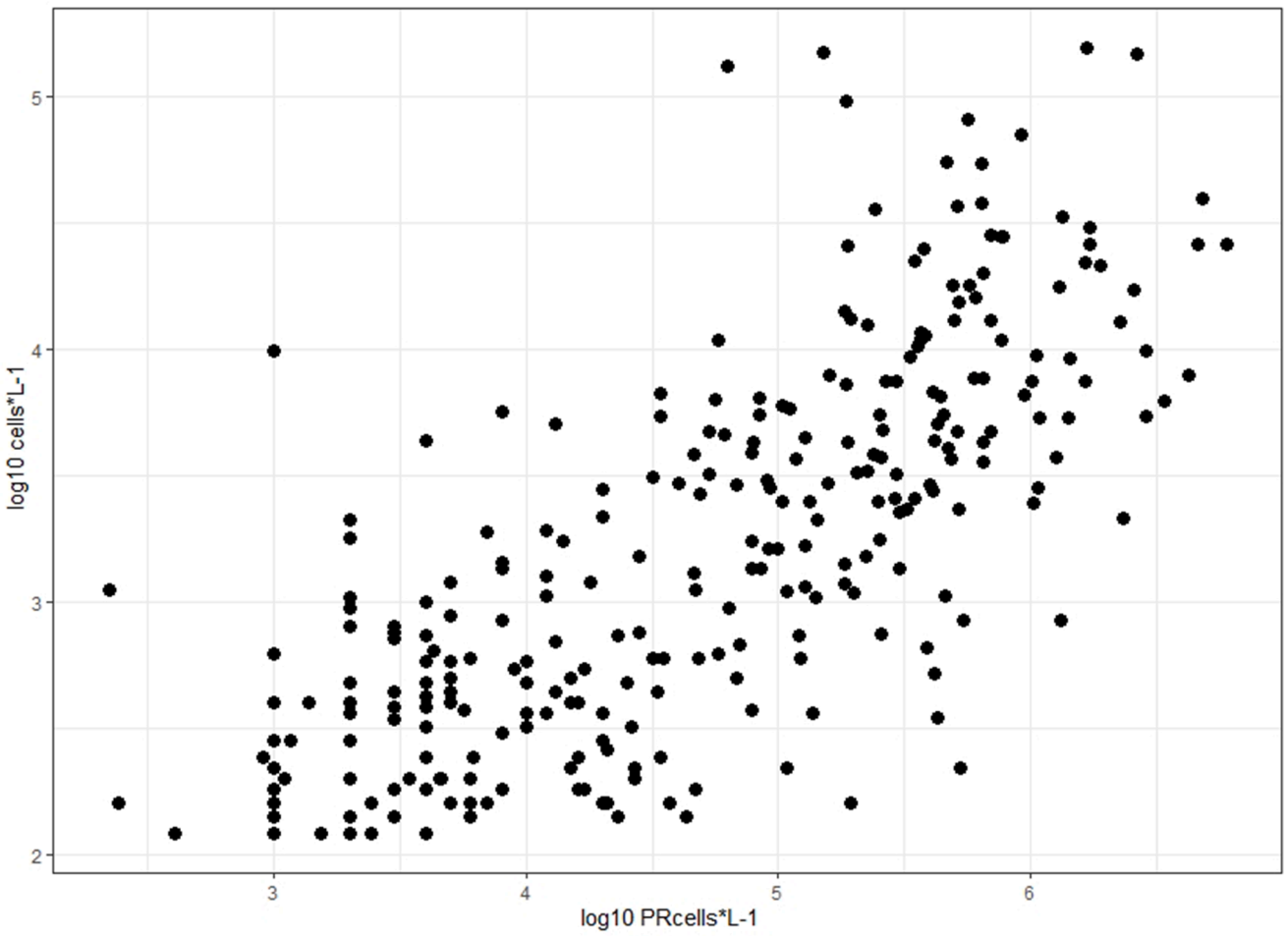

Fig. 7. Correlation between $\log_{10}$ Potentially Resuspended cells (PRcells·L^{-1}) and $\log_{10}$ PLK cells (cells·L^{-1}), for PLK cell concentrations over 100 cells·L^{-1}.

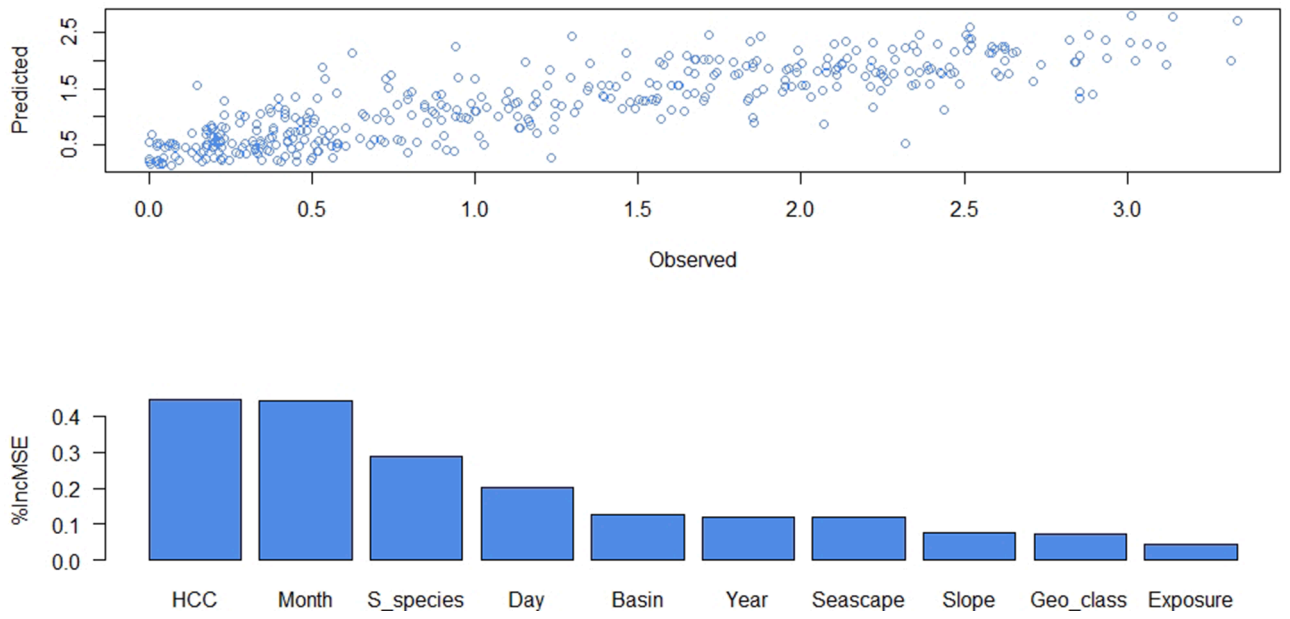

Fig. 8. Correlation between observed and predicted $\log_{10}$ BEDI cells (cells·mm^{-2}), according to Random Forest Model (a); barplot of the importance of each feature used in the Random Forests Model in terms of percentage of increase in mean square error (MSE) when out-of-bag data for that variable were permuted while all others were left unchanged (b).

not EPI and BEDI abundances. This means that the proliferation of the cells (the benthic stock, *sensu* Mangialajo et al., 2017) is not affected by topographic features, but small-scale water movement (currents, water retention) may significantly determine detachment of cells from the substrate.

The present large-scale comparison of PLK, EPI and BEDI samples allowed to test correlations among cell abundances assessment by the different approaches on a large number of samples and across a wide range of environmental settings. Cell abundances are generally accounted for by collecting water samples, an approach that is independent *per se* of the specific substrate collected and therefore may be generally implemented and allows comparisons across sites. Such an approach is assumed to accounting for the cells that may directly affect humans by direct contact and inhalation (e.g., Tubaro et al. 2011; Funari et al., 2015). However, the PLK cell concentration is also highly variable and PLK cell assessment is time consuming, since the (usually) low concentrations in the water column require overnight sedimentation through 50 ml Utermöhl chambers. But, most of all, the PLK cell assessment approach does not account for the actual number of stock cells, since *Ostreopsis* spp. are benthic and proliferate on the sea bottom.

As mentioned, the estimation of microalgal cells collected on biotic substrates is highly affected by the specific macroalgal species considered, in particular, as a function of the macroalgal weight, shape, surface provided and, potentially, chemical interactions (e.g., Blanfuné et al., 2015; Ternon et al., 2021; Monserrat et al., 2022). In addition, the assessment of benthic cell abundances requires the direct collection of the substrates, including biotic substrates. This approach is inherently destructive and should not be considered sustainable for long term monitoring plans when canopy forming species (or corals, in the tropical latitudes) need to be collected. Therefore, different approaches have been implemented to account for the benthic *Ostreopsis* cells (or other B-HAB taxa) on biotic substrates, which allow non-destructive samplings and performing comparisons among different benthic communities and habitats (e.g., Tester et al., 2014; 2022).

Among them, the BEDI approach first proposed by Mangialajo et al. (2017) and applied in the current paper provides the first large-scale assessment of its effectiveness compared to PLK and EPI cell abundance estimation. Cell concentrations estimated by BEDI (in terms of cells·mm^{-2}), and EPI samples (in terms of cells·gFW^{-1}) showed a very good correlation (Fig. 4) across the different environments. This relationship turned out to be consistent across spatial (Fig. 5) and temporal (Fig. 6) scales, supporting the broad potential of BEDI device use. Remarkably, the differences accounted for the different macroalgal sampled species for the EPI cell abundance assessment were maintained when using the BEDI sampling: indeed, the epiphytic *O.* cf. *ovata* concentrations estimated in EPI samples collected on *Ellisolandia elongata, Hypnea musciformis* and *Padina pavonica* displayed lower values than on other sampled macroalgae; this trend was also observed in the integrated BEDI samples collected on habitats dominated by these macroalgal species.

BEDI samples, in terms of Potentially Resuspended (detached) cells (PRcells·L^{-1}), displayed also a remarkably good correlation with PLK samples (Fig. 7), enhancing the suitability of this approach also for assessing the actual potential risk of human exposure to toxic microalgae.

Therefore, BEDI can be considered a good proxy for estimating *Ostreopsis* cells stock in different environmental settings, and also for assessing the potential threat for humans, particularly in areas where trampling and swimming can enhance cell detachment from the benthic substrate. In this sense, the BEDI method may constitute an integrative approach, since it accounts for the sum of PLK cells and cells that can be detached from the substrate: therefore, it really accounts for the whole potential exposure for both marine organisms and human beings. In addition, the BEDI method is fast (since the usually high cell abundance samples collected do not require the sedimentation chamber step necessary for the estimation of PLK cells concentrations), non-destructive and can provide near real time cell abundance assessments, that well reflect the risk of human exposure to aerosols, assessing the potentially resuspended cells, integrating both the cells in the water and the ones attached to the substrate.

Of course, the method also has some drawbacks, as pointed out in Mangialajo et al. (2017): e.g., the prototype is difficult to handle by only one person, especially in relatively rough sea conditions. Nevertheless, a consistent agreement was found between the integrated cell abundances estimated by BEDI and both plankton and epiphytic cell numbers. These findings support the use of BEDI as a non-destructive sampling device, an essential question in the present context on global biodiversity and habitat loss with direct effect on *Ostreopsis* spp. expansion and blooms. Our study has also confirmed that the loss of high complexity communities, i.e., macroalgal forests, may increase the risk of *Ostreopsis* blooms, enforcing the need to protect these essential ecosystems.

CRediT authorship contribution statement

Mariachiara Chiantore: Writing – review & editing, Writing – original draft, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Valentina Asnaghi:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Marie Abboud-Abi Saab:** Writing – review & editing, Investigation, Data curation. **Laury Acaf:** Writing – review & editing, Data curation. **Stefano Accoroni:** Writing – review & editing, Investigation, Data curation. **Ali Badreddine:** Writing – review & editing, Investigation, Data curation. **Laura Escalera:** Writing – review & editing, Investigation, Data curation. **Anna Fricke:** Writing – review & editing, Investigation, Data curation. **Cécile Jauzein:** Writing – review & editing, Methodology, Investigation, Data curation. **Rodolphe Lemée:** Writing – review & editing, Methodology, Investigation, Data curation. **Cecilia Totti:** Writing – review & editing, Investigation, Data curation. **Souad Turki:** Writing – review & editing, Investigation, Data curation. **Magda Vila:** Writing – review & editing, Investigation, Data curation. **Imen Zaghmourii:** Writing – review & editing, Investigation, Data curation. **Adriana Zingone:** Writing – review & editing, Investigation, Data curation. **Elisa Berdalet:** Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Luisa Mangialajo:** Writing – review & editing, Writing – original draft, Methodology, Data curation, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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