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Management of harmful benthic dinoflagellates requires targeted sampling methods and alarm thresholds



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16

17 **Abstract**

18 Concern regarding Benthic Harmful Algal Blooms (BHABs) is increasing since some harmful
19 benthic species have been identified in new areas. In the Mediterranean basin, the most common
20 harmful benthic microalgae are *Ostreopsis* cf. *ovata* and *Prorocentrum lima*, which produce
21 palytoxin-like compounds and okadaic acid respectively, and the need to implement monitoring
22 activities has increased. However, a general agreement on appropriate strategies (e.g. sampling
23 season, definition of alarm thresholds, etc.) is still lagging behind, especially for *P. lima*, whose
24 proliferation dynamics are still poorly known.

25 The aim of this study is to highlight similarities and differences between these two benthic
26 dinoflagellates, providing a six-year time series of their co-occurrence in relation to environmental
27 variables (in particular, hydrodynamic sea conditions). Our data report species specific effects of
28 the hydrodynamic regime in terms of affinity of microalgal cells with the benthic substrate,
29 suggesting that *P. lima* has a more strictly "benthic behavior" compared to *O. cf. ovata*.

30 These results highlight the particular need to implement a specific sampling strategy and define
31 benthic alarm thresholds, triggering future applications in a management perspective.

32 **1. Introduction**

33 Benthic Harmful Algal Blooms (BHABs) refer to the proliferation of benthic microalgae, which may
34 represent a serious threat for other organisms (including humans) and the environment. In
35 temperate regions, BHABs started receiving large interest in the last decade, since certain harmful
36 benthic species, mainly of tropical origin, have been identified in new areas, gaining the attention of
37 both the scientific community and public governance.

38 As an example, *Ostreopsis* spp. have been recorded in many locations along the Mediterranean
39 (Accoroni et al., 2016), Australian (Verma et al., 2016), New Zealand (Shears and Ross, 2009) and
40 Japanese coasts (Parsons et al., 2012). This genus is responsible for the Palytoxicosis (foodborne
41 poisoning) and other irritative symptoms (through direct contact and aerosols), respectively in the
42 tropics and in temperate areas (Berdalet et al., 2015).

43 The genus *Gambierdiscus* has been reported in Micronesia - Macaronesia (Pérez-Arellano et al.,
44 2005), Mediterranean Sea (Aligizaki et al. 2008), Canary Islands (Fraga et al. 2011), Japan
45 (Nishimura et al., 2013 and 2014) and The Cook Island (Smith et al., 2016); this genus is involved
46 in the Ciguatera Fish Poisoning, an endemic human syndrome in tropical and subtropical areas,
47 presently spreading to temperate latitudes.

48 Species belonging to the genus *Prorocentrum* spp., and especially *Prorocentrum lima*, are more
49 cosmopolitan than the other BHABs presenting a larger geographical distribution (Aligizaki et al.
50 2009); some *Prorocentrum* spp. are responsible for Diarrheic Shellfish Poisoning (DSP) (Berdalet
51 et al., 2015).

52

53 In the Mediterranean Sea, the most common benthic harmful dinoflagellates are *Ostreopsis* cf.
54 *ovata* and *P. lima*.

55 The former species lives associated with a variety of biotic and abiotic substrata (macroalgae,
56 seagrasses, benthic invertebrates, sand, pebbles and rocks), attached through mucilaginous
57 filaments, which lump together microalgal cells (Totti et al., 2010). In general, *O. cf. ovata*
58 proliferates during the summer season, from June to September (except for the Adriatic Sea,
59 where the maximum abundance is usually reached in October; Accoroni et al., 2012), when sea
60 temperature exceeds 24 °C, especially in sheltered and shallow-water areas (Mangialajo et al.,
61 2008; Pezzolesi et al., 2012). During long lasting blooms, its mucilaginous matrix can form a
62 brownish mat that can easily be re-suspended by mechanical action or local hydrodynamics:
63 consequently, the concentration of *O. cf. ovata* in the water column is related to its abundance in
64 the benthic habitat (Mangialajo et al., 2011).

65 This dinoflagellate occurs in almost all Italian coastal waters (ISPRA, 2013) and produces a wide
66 range of palytoxin-like compounds (PLTXs) (Ciminiello et al., 2010, 2012a and 2012b), which can
67 affect both humans and marine organisms. Data related to *Ostreopsis* spp. toxins and their effects
68 are reported in several studies (e.g. Deed and Schwartz, 2010; Ramos, 2010; Alloisio et al., 2016).
69 Considering all available data on *O. ovata* toxicity and the Acute Reference Dose (ARfD: amount of
70 substance that can be consumed over a short period of time without any appreciable health risk for
71 consumers) set by the European Food Safety Authority (ESFA) at 0.2 µg of PLTXs equivalent / kg
72 of body weight, the Italian National Institute of Health has recently updated the guidelines for
73 assessing and managing human health risks associated to *Ostreopsis* (Funari et al., 2014; 2015).
74 According to these guidelines, monitoring activities are planned according to observed bloom
75 phase (Routine, Alert and Emergency). In particular, the "Alert status" starts when the cell density
76 of *O. cf. ovata* in the water column is between 10 000 and 30 000 cells L⁻¹, with a prolonged period
77 (7-10 days) of low hydrodynamic conditions and high temperature.

78
79 Knowledge on *P. lima* proliferation dynamics, conversely, is very limited, although this species has
80 been frequently recorded in several Mediterranean areas (Catalan coasts, Spain: Vila et al., 2001;
81 North Aegean Sea: Aligizaki et al., 2009; Italian coasts: Simoni et al., 2004; Ingarao et al., 2007;
82 Cyprus: author's unpublished data). In general, it shows a golden-brown chloroplast and a centrally
83 located pyrenoid in each valve view, smooth theca and valve and marginal pores, slightly visible by
84 light microscope; however it displays a wide variability in cell shape and size (Aligizaki et al. 2009).
85 Due to its production of okadaic acid (OA), along with other DSP toxins, *P. lima* induces diarrheic
86 symptoms by acting as a potent protein phosphatase inhibitor in metabolic processes; chronic
87 exposure to these toxins is hypothesized to stimulate gastrointestinal tumors (Aune & Yndestad,
88 1993). Regarding toxicity of the Mediterranean *P. lima* strains, there is lack of information and, so
89 far, no official alarm threshold (in terms of cell concentration) has been established in this region.

90 However, OA-group toxin levels are monitored in Europe and food enterprises must ensure that
91 bivalve molluscs placed on the market for human consumption do not exceed the current
92 regulatory limit of 160 µg of OA kg⁻¹ shellfish meat and the Acute Reference Dose fixed by EFSA is
93 0.3 µg kg⁻¹ body weight. Some European countries recently adopted the threshold of 100 cells L⁻¹
94 (Ireland, UK) or 500 cells L⁻¹ (Mediterranean coast of Spain) to activate more accurate monitoring,
95 for example, before the harvesting and sale of aquaculture products (ICES Report, 2015).

96
97 Monitoring of benthic microalgae in the Mediterranean basin recently increased due to the related
98 concerns and, so far, the most common applied strategy is based on seawater sampling and, in a
99 few cases, on macrophyte collection: herein estimation of microalgal species abundances is
100 assessed through light microscopy after detachment from the substrate. While *Ostreopsis*
101 monitoring has been widely discussed and some projects focused on sampling and counting
102 optimization (e.g. www.m3-habs.net), a general agreement on appropriate strategies (e.g.
103 sampling season, definition of alarm thresholds) is still lagging behind, especially regarding other
104 potentially toxic species, such as *P. lima*.

105 Being the quantification of benthic microalgae more difficult to standardize, due to the spatial and
106 temporal scales of variability of the benthic system, different sampling approaches have been
107 tested (see GEOHAB 2012; Tester et al., 2014; Jauzein et al., 2016). The lack of an appropriate
108 and standardized sampling approach may be responsible for problems in properly measuring of
109 their abundances and perform comparisons across locations.

110 The aim of this study, is to provide data on proliferation dynamics of *P. lima* along Genoa coast
111 (Italy, North-western Mediterranean Sea), highlighting similarities and eventual differences
112 compared to *O. cf. ovata* in terms of "ecological behavior", which could push to the adoption of
113 specific and more targeted sampling strategies, using a six year monitoring data set.

114

115 **2. Materials and Methods**

116 2.1. Sampling strategy

117 Data presented in this study refer to the BHABs monitoring campaign carried out at Quarto dei
118 Mille (Genoa, Italy) during the summer seasons from 2010 to 2015, for a total of 87 sampling
119 occasions: from June 24th to 1st of September 2010, from June 14th to August 31th 2011, from June
120 20th to October 10th 2012, from June 7th to September 23th 2013, from June 13th to September 9th
121 2014 and from June 18th to 10th September 2015.

122 Sampling was performed by collecting seawater samples and macrophytes (the brown alga
123 *Halopteris scoparia*), following the commonly applied protocol(as reported e.g. in Mangialajo et al.,

124 2011. Sampling was performed in the morning once a week, incremented to 2-3 times per week
125 during the bloom period, keeping a similar sampling time (from 8:00 to 10:00 am) to avoid
126 fluctuations due to potential diel variations between benthic and planktonic pools. Planktonic
127 samples (1 liter of seawater, in duplicate or triplicate) were collected at about -0.3m depth, before
128 collection of benthic ones (5-10 g fresh weight of algae, at -0.5 m deep) in order to avoid a
129 mechanical re-suspension of benthic cells. Samples were fixed with formalin (2%) from 2010 to
130 2013 and with acidic Lugol solution followed by storage at 4°C in 2014 and 2015, according to an
131 improved and standardized protocol developed in the framework of the M3-HABs Project
132 (<http://m3-habs.net>).

133 Cells abundance in seawater samples was assessed using the Utermöhl sedimentation method
134 (100 ml column settled for 24 h). In regard to benthic samples, the protocol was slightly changed in
135 2014 adding a rinsing step after the shaking procedure used to detach epiphytic cells. From 2010
136 to 2013 macrophytes and surrounding seawater were vigorously shaken to detach benthic cells
137 and 3 ml of seawater were settled in the Utermöhl chamber. In 2014 and 2015, isolation of
138 epiphytic cells was performed by vigorously shaking (for 10 sec) the macrophyte samples, then
139 rinsing with 100 ml of filtered seawater (0.2 µm) and shaking two times. Counting was performed
140 on 1 ml, using the Sedgewick Rafter chamber.

141 Abundances of dinoflagellates were expressed as cells g⁻¹ fresh weight of macrophyte (fwm) in the
142 case of benthic samples and in cells L⁻¹ for seawater ones. Identification of the two species was
143 carried out through light microscopy.

144 2.2. Environmental data

145 Seawater temperature and salinity data were recorded *in situ* using a CTD probe (Hanna 9328),
146 concurrently with sample collection. Data regarding sea condition, reported as days with calm sea
147 (wave height < 0.5 m) or rough sea (waves > 0.5 m), have been provided by DHI srl
148 (<https://worldwide.dhigroup.com/it>).

149 2.3. Data and statistical analyses

150 Alarm thresholds for *O. cf. ovata* used in this work were defined according to the Italian guideline
151 2014 (10 000 - 30 000 cells L⁻¹, according to Funari et al., 2015). As far as *P. lima*, the threshold
152 concentration of 500 cells L⁻¹ was taken into account, as from the ICES Report 2015.

153 Correlation analyses (separately for both target species) were performed between microalgal
154 densities in the water column and on macrophytes to investigate the effects of sea conditions on
155 the re-suspension of cells from the substrate. In addition, for assessing the potential different
156 behavior of *P. lima* and *O. cf. ovata* in response to the hydrodynamic regime, the ratio between
157 concentration in the water column and on macrophytes (cells L⁻¹ versus cells g⁻¹ fwm) was plotted

158 separately for calm (wave height <0.5 m) or rough sea-conditions (wave height > 0.5 m for more
159 than 6 hours in the two days before sampling). Analysis was performed only on data collected
160 during blooming periods of 2010, 2011, 2012, 2013 and 2015 (from 1st of July to August 15th; 56
161 total sampling occasions). Summer 2014 has been excluded due to the unavailability of wave
162 height data. Statistical analyses were carried out using the software R (R Development Core
163 Team, 2008).

164 3. Results

165 3.1. Monitoring data

166 Maximum cell density values of *O. cf. ovata* and *P. lima* and related alarm thresholds, seawater
167 temperature and hydrodynamic conditions are summarized in Table 1. Concentrations of both
168 species both in the water column and epiphytic on *H. scoparia*, recorded during 2010, 2011, 2012,
169 2013, 2014 and 2015 summer seasons are plotted in Fig. 1 and 2.

170 *Ostreopsis cf. ovata* reached the highest abundance in the water column (51 719 cells L⁻¹), as well
171 as on macrophytes (2 289 100 cells g⁻¹ fwm), during summer 2015, which was characterized by
172 notably good weather and calm sea conditions. In fact, in summer 2015 the average seawater
173 temperature (25.9 °C) and percentage of days with calm sea (75 %) were higher than in the other
174 years. On the contrary, the lowest peak concentration in the water column was recorded in 2011 (4
175 770 cells L⁻¹), in conjunction with only 42.3 % of days with calm sea and quite low average
176 seawater temperature (24.7 °C). Lowest abundance on macrophytes was recorded in summer
177 2014, which was again characterized by comparatively low temperatures (23.8 °C) and unstable
178 sea conditions (authors observation). Alarm threshold of this species (> 30 000 cells L⁻¹) was
179 exceeded on three days during 2010, one day in 2012, 4 days in 2013 and one day in 2015.

180 Also *P. lima* achieved the highest concentrations in 2015, reaching 530 cells L⁻¹ in the water
181 column and 74 176 cells g⁻¹ fwm. Conversely, lowest maximum densities in the water column
182 occurred in summers 2010 and 2013 (141 and 115 cells L⁻¹ respectively). Threshold was exceeded
183 only once in 2015.

184 In general, taking into account both species, no relevant health problems nor impacts on the
185 marine fauna were recorded in the studied area during the years considered.

186 3.2. Proliferation dynamics and ecological behavior

187 Significant correlations between cells in the water column and on macrophytes were reported for
188 both species ($p < 0.001$ for both *O. cf. ovata* and *P. lima*) but the *O. cf. ovata* model was notably
189 stronger ($R^2 = 0.79$) than the one for *P. lima* ($R^2 = 0.15$). Cell densities recorded with calm or rough
190 sea conditions (see MM section for definition) in the water and on the macrophytes did not show

191 species specific patterns according to sea conditions (Fig. 3), although species specific effects of
192 the hydrodynamic regime can be inferred in terms of affinity of microalgal cells with the benthic
193 substrate (Fig. 4). In particular, the ratio between planktonic and benthic cells of *O. cf. ovata* is
194 more variable in both calm and rough sea conditions, suggesting that its cells more autonomously
195 move from the substrate to the water column (regardless of seawater hydrodynamics). On the
196 other hand, data regarding *P. lima* show lower ratio between planktonic and benthic (so less
197 specimens occur in seawater relative to concentration on the benthic substrate), no sea condition
198 effects and lower variability within sea conditions (calm vs rough), suggesting a strictly "benthic
199 behavior" and a high degree of attachment to the benthic substrate.

200

201 4. Discussion

202 The epiphyte dinoflagellate assemblage constituted by *Ostreopsis* spp., *P. lima*, *C. monotis* is a
203 well known group of species, frequently co-occurring in the Mediterranean Sea (Tindall & Morton
204 1998; Vila et al., 2001). While *Ostreopsis* species have been more regularly monitored in different
205 Mediterranean areas and data regarding *O. cf. ovata* in terms of cell abundances and distribution
206 (Mangialajo et al., 2011, Penna et al., 2010), ecology (Accoroni et al., 2012; Asnaghi et al., 2012;
207 Carnicer et al., 2015), toxins content (Dell'Aversano et al., 2014) and toxic effects (Giussani et al.,
208 2016 and references within) are becoming more conspicuous, the other species still need to be
209 more comprehensively addressed.

210 In the framework of the present study, a 6-year time series of summer monitoring data on
211 *Ostreopsis cf. ovata* and *Prorocentrum lima*, both epiphytic on macroalgae and suspended in
212 seawater, and related environmental variables (in particular seawater temperature and
213 hydrodynamic conditions) were collected, showing that summer proliferations of *O. cf. ovata* and *P.*
214 *lima* commonly occur along the Genoa coast (Ligurian Sea, Italy).

215 *O. cf. ovata* showed a clear intra-seasonal trend, comparable among the different years and similar
216 for cells sampled in seawater and on macroalgae, with peaks of abundance recorded between mid
217 July and the beginning of August, as also previously observed in other NW-Mediterranean areas
218 (Mangialajo et al., 2011). In years characterized by a steep increase in seawater temperature, with
219 heat peaks after the first-half of July, proliferation trends were more evident compared to years (i.e.
220 2011 and 2014) when seawater temperatures remained lower and more constant along the
221 summer season (Fig. 1 and 2), confirming the relevant role of seawater temperature rise in driving
222 bloom events, as already highlighted by laboratory (Manganelli et al., 2007; Graneli et al., 2008),
223 field (Mangialajo et al., 2008; Totti et al., 2010; Cohu et al., 2013) and model (Asnaghi et al., 2017)
224 studies.

225 On the contrary, *P. lima* did not show any particular pattern related to seawater temperature,
226 neither for cells recorded in seawater nor on macroalgae. Cells on macroalgae and in seawater
227 showed quite stable abundances during the sampling periods across the considered years, without
228 any particular detectable intra-seasonal trend (Figs. 1 and 2).

229 Similar findings on the relative abundance of the two species are provided by another monitoring
230 study performed throughout the whole year along the Catalan coast from July 1997 to July 1998
231 (Vila et al., 2001). This study showed that higher densities were reached during warmer months. In
232 particular, maximum recorded abundance of *P. lima* was around 700 cells g⁻¹ fwm (and only few
233 cells in seawater) but the dominant species was always *Ostreopsis* sp. in all investigated media
234 (seawater, macrophytes and sand), which reached the highest density of 596 000 cells g⁻¹ fwm in
235 July 1997. On the contrary, in a recent study reporting a massive proliferation of *P. lima* (about 45
236 000 cells g⁻¹ fwm) during summer 2011 along French Catalan coast and Languedoc, *Ostreopsis*
237 spp. was not found (Blanfuné et al. 2015). Higher abundances of the *Prorocentrum* group and a
238 slightly delayed proliferation period was reported by a two-year monitoring study performed along
239 the North Aegean Sea (Aligizaki et al., 2009), which carried out a monthly survey from August
240 2003 to December 2005. "*P. lima* species-complex" was the most common epiphytic species
241 (among *Prorocentrum* genus), reaching relatively high densities both on macrophytes (133 167
242 cells g⁻¹ fwm on *Cymodocea nodosa* in November 2005) and in seawater (11 280 cells L⁻¹ at the
243 end of October 2003). This species-complex was recorded all year round, with a temperature
244 range from 10.0 to 29.5 °C, but maximum abundances occurred during late autumn. In the same
245 study, other dinoflagellate toxins producers belonging to the *Prorocentrum* group were reported to
246 bloom during the summer season: *P. levis* (Faust et al. 2008; up to 918 cells g⁻¹ fwm in late June
247 or early July) and *P. borbonicum* (Ten-Hage et al. 2000; up to 942 cells g⁻¹ fwm in August –
248 September), both found almost only on benthic samples.

249 The alarm threshold range considered for *O. cf. ovata* (10 000 - 30 000 cells L⁻¹) was exceeded in
250 several occasions during the study period (Table 1), with the exception of 2011 and 2014, while *P.*
251 *lima* exceeded the set threshold only once (500 cells L⁻¹) in 2015. It is noteworthy that in 2015, an
252 actual much larger bloom of *P. lima* on macroalgae occurred (average cells g⁻¹ fwm around 10 fold
253 higher than in the other years), but such remarkable increase was not equally mirrored by the
254 concentration in seawater.

255 The different ratio between concentration in seawater and on macroalage displayed by the two
256 microalgae (Fig. 4) suggests a quite different behavior of the two species, independently from
257 hydrodynamic conditions. In fact, although both species are benthic, *O. cf. ovata* is much more
258 prone to move from the substrate to seawater, and a general good correlation between the two
259 compartments has been observed in the present study (Fig. 3a) as well as in previous ones
260 (Magialajo et al., 2011). As a consequence of this behavior, setting alarm threshold for the cell

261 concentration in seawater for *O. ovata* is actually meaningful, because the concentration in
262 seawater is closely mirroring the one on macroalgae. Differently, for *P. lima* the threshold set in
263 seawater seems not to be representative of the actual proliferation occurring on the substrate,
264 suggesting the need for the definition of targeted benthic thresholds.

265 Similarly, *P. lima* behavior in the Gulf of Maine (Northeastern USA coast), has been described
266 emphasizing its characterization as an epiphytic or benthic dinoflagellate, and strengthening the
267 argument that monitoring techniques designed for the water column would be ineffective in this
268 case (Maranda et al. 2007).

269 It is noteworthy that *P. lima*, given its strictly benthic behavior, is expected to be less dangerous for
270 human health, in terms of accidental ingestion or inhalation of aerosols; nevertheless, it could
271 become an issue for aquaculture activities due to the high density that it can reach in fouling
272 macroalgal biomass around mollusc lines (Maranda et al. 2007a). Additionally, it is noteworthy that
273 the toxin level is not always correlated with the mere number of algal cells (Maranda et al. 2007b;
274 Vannucci et al. 2010).

275 An appropriate sampling strategy, specifically designed for benthic species (e.g. collection of
276 natural or artificial substrates), is necessary in order to accurately assess potential threatening
277 effects of *P. lima*, more than for other benthic microalgae, such as *Ostreopsis*.

278 Providing a six-year time series of the co-occurrence of *P. lima* and *O. cf. ovata* and their
279 ecological behavior in relation to environmental variables, the present study highlights the
280 particular need to implement a specific sampling strategy and define benthic alarm thresholds for
281 strictly benthic microalgae, such as *P. lima*, triggering future applications in a management
282 perspectives.

283

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290

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490 **Figure legends**

491 Figure 1. Densities (\pm sd) of *O. cf. ovata* (black) and *P. lima* (grey) in the water column and on the
492 macrophyte *H. scoparia* during summer seasons 2010, 2011 and 2012 at Quarto dei Mille, Genoa
493 (Italy). Dotted line indicates seawater temperature. Dark-gray shade highlights *O. cf. ovata*
494 threshold range and light-gray dotted line indicates *P. lima* threshold.

495 Figure 2. Densities (\pm sd) of *O. cf. ovata* (black) and *P. lima* (grey) in the water column and on the
496 macrophyte *H. scoparia* during summer seasons 2013, 2014 and 2015 at Quarto dei Mille, Genoa
497 (Italy). Dotted line indicates seawater temperature. Dark-gray shade highlights *O. cf. ovata*
498 threshold range and light-gray dotted line indicates *P. lima* threshold.

499 Figure 3. Correlation between *O. cf. ovata* (left; $p < 0.001$ and $R^2 = 0.79$) and *P. lima* (right; $p <$
500 0.001 and $R^2 = 0.15$) cell concentrations in seawater (cells L^{-1}) and on macrophyte (cells g^{-1} fwm).
501 Circles refer to data collected in calm sea conditions, triangles to wave height > 0.5 m.

502 Figure 4. Boxplots of ratio between cells in seawater and on macrophyte (*O. cf. ovata* on the left; *P.*
503 *lima* on the right) in calm and rough ($<$ or > 0.5 m high wave; see MM section) sea conditions,
504 during the blooming periods (1st of July – August 15th of 2010, 2011, 2012, 2013 and 2015).

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