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1 **Title:** Seasonal patterns of settlement and growth of introduced and native ascidians in
2 bivalve cultures in the Ebro Delta (NE Iberian Peninsula)

3
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15
16 **Abstract**

17 Ascidians are important both as invasive species and as a fouling group in artificial marine
18 habitats, causing negative impacts in aquaculture settings and the surrounding
19 environment. The Ebro Delta is one of the major centres of bivalve production in the
20 Mediterranean and is affected by proliferation of ascidian species (mostly introduced
21 forms). Knowledge of the patterns of settlement and growth of the fouling species is
22 mandatory to attempt mitigation measures. We deployed settlement PVC plates from May
23 to September 2015 at different depths (0.2, 1 and 2 m) in the Ebro Delta oyster
24 aquaculture facilities. We then monitored the occurrences of all species and the area cover
25 of a selected subset of 6 species on a monthly basis from June 2015 to December 2016.
26 We found 15 species, of which 10 are introduced. There were some differences between
27 our plates and the oyster ropes in species abundance and composition, likely due to
28 differences in substrate complexity. For instance, *Didemnum vexillum* and *Clavelina*
29 *oblonga* occurred in few plates in contrast to their abundance on oysters. The most
30 abundant species were *Styela plicata* and *Clavelina lepadiformis*, which together with
31 *Ecteinascidia turbinata* showed a preference to grow on plates deployed in May and June.
32 Most of the species grew more at 0.2 m depth than at deeper plates. Thus, to minimise
33 fouling on bivalves, we propose spat immersion during fall and below 1 m depth. We also
34 found that number of occurrences and cover of the species are similarly informative; we
35 suggest that a periodic monitoring of species occurrence on replicate plates is sufficient for
36 detecting new introduced species as soon as possible and will provide information useful
37 for management.

38
39 **Keywords:** aquaculture facilities; invasive species; ascidians; fouling; *Styela plicata*,
40 *Didemnum vexillum*.

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42

43 **1.Introduction**

44 Ascidians are among the most important fouling groups in man-made marine habitats
45 (Aldred & Clare 2014). In particular, they pose important problems in aquaculture facilities
46 where they can become a dominant group (Fitridge et al. 2012). At the same time,
47 ascidians are well-known for their many important invasive species (Lambert 2007). Both
48 aspects are inextricably linked, as artificial structures favour the spread of introduced
49 ascidians (Simkanin et al. 2012; Airoidi et al. 2015; López-Legentil et al. 2015), being one
50 of the principal pathways of marine invasions (Naylor et al. 2001). Thus, biofouling by
51 ascidians often leads to explosive growth of some species and detrimental effects in
52 aquaculture settings, causing both economic and ecological negative impacts (Carver et
53 al. 2003; Blum et al. 2007; Lutz-Collins et al. 2009).

54 In bivalve cultures, ascidians add weight and compete with the farmed species for food
55 resources, which translates into a higher bivalve mortality and a lower overall size, thus
56 decreasing bivalve productivity (Daigle & Herbinger 2009). Knowledge of the settlement
57 and growth cycles of ascidians is mandatory for their management, and particularly so in
58 aquaculture settings where seasonality of the farming can interact with the seasonality of
59 the fouling species themselves (Daigle & Herbinger 2009; Valentine et al. 2009). This
60 knowledge should be acquired *in situ*, analysing the local populations, as phenotypic
61 plasticity and adaptation generate shifts in life history traits of ascidians (Wagstaff 2017). In
62 addition, their effects are also context-dependent (Robinson et al. 2017), thus rendering
63 studies in areas other than the ones affected of little utility.

64 The Ebro Delta (NE Iberian Peninsula) is one of the major centres of bivalve aquaculture
65 in the W Mediterranean, with a production of about 4,000 tons per year. Recently,
66 proliferation of newly introduced ascidians such as *Clavelina oblonga* and *Didemnum*
67 *vexillum*, have been reported in the area (Ordóñez et al. 2015, 2016). These have added
68 to the previous presence of introduced and native ascidians (Turon 1987; Perera et al.
69 1990) resulting in heavy fouling on the bivalves, and concomitant negative impacts.

70 The goal of this study is to analyse the diversity and temporal dynamics of ascidians in the
71 Ebro Delta oyster culture facilities. We deployed settlement panels over spring and
72 summer 2015 and monitored them regularly for 20 months. We assessed the role of
73 deployment date, depth, and seasonality on the presence and abundance of ascidian
74 species. Our final goal was to generate basic information useful for minimising losses in
75 bivalve production due to ascidian overgrowth.

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78 **2.Material & Methods**

79 The study site was located in Fangar Bay, at the northern side of the Ebro Delta in the NE
80 Iberian Coast (40°46'27.43"N, 0°44'27.11"E, Figure 1). Fangar Bay has 9 km² of surface
81 area with a muddy bottom up to 4.2 m depth. Bivalve rafts are used to grow the oyster
82 *Crassostrea gigas* and the mussel *Mytilus galloprovincialis*. Each raft is supported by
83 cement pilings and consists of a rectangular structure of wooden beams arranged in a
84 grid, from which the bivalve ropes hang.

85 We used a raft located in the middle of the aquaculture facilities to hang a total of 15 ropes
86 with settlement plates. All ropes were placed on the same side of the raft and were
87 interspersed along its length. From May to September 2015, 3 ropes were placed each
88 month and left in place until the end of the study. On each rope, 3 PVC plates pre-
89 roughened with coarse sandpaper, 20x20 cm in size, were vertically attached with tie-

90 wraps, at 0.20, 1 and 2 m depth, respectively.

91 The ropes were examined twice per month from June 2015 to August 2015, and once per
92 month from September 2015 to December 2016. Photos from each side of all plates were
93 taken, and notes about the ascidian species present were recorded *in situ*. The occurrence
94 of a species in a given period of time was defined as the total number of plates where the
95 species was present. Some samples were collected on the oyster ropes (to avoid
96 interference with the study), formalin-preserved, and examined in the laboratory to verify
97 the identity of species using taxonomic characters. The photos were used to calculate the
98 areas covered by a selected group of ascidians using the program Fiji (Schindelin et al.
99 2012).

100 Cover was calculated as the percent of the total area of both sides of a given plate
101 occupied by a given species. Although the architecture of some species was not perfectly
102 two-dimensional, the fouling on the plates showed in general a low vertical development.
103 Thus, area measurements served as an adequate proxy for species' growth. Percent cover
104 values were analysed using profile analysis (Quinn & Keough 2002). In this approach to
105 repeated measures analysis, the variable of interest (cover) is integrated over time and the
106 resulting value is used to test the relevant factors. As response variable, we used the
107 integral of the cover values over time (calculated with R, R Core Team 2015). The factors
108 considered were: initial date (i.e. the five immersion dates, fixed), depth (three levels,
109 fixed), and rope as a blocking factor (random) nested within date. The model was tested
110 using a randomization procedure in PERMANOVA (Anderson et al. 2008) with Euclidean
111 distance to construct the resemblance matrix and 999 permutations of the data.

112 We ran separate analyses for each species and season, starting in fall 2015. Area cover
113 values were integrated only over the season of interest. We also ran an analysis
114 considering the whole studied period, integrating cover values over all observation times.
115 The graphics were plotted using the R package "ggplot2" (Wickham 2009) and SigmaPlot
116 v.12 (Systat Software, San Jose, CA, USA).

117 Additionally, water temperature ($^{\circ}\text{C}$), salinity (psu), dissolved oxygen percent (%) and
118 chlorophyll *a* concentrations ($\mu\text{g L}^{-1}$) were obtained during the monitoring period from
119 weekly measurements of the long-term monitoring program of the Institute of Agriculture
120 and Food Research and Technology (IRTA). Environmental variables are presented as
121 monthly means. We ran cross-correlation analyses relating the cover values of the
122 ascidian species with the values of these variables during the same and the three previous
123 months using Systat v.12 (Systat Software, San Jose, CA, USA).

124

125

126 **3.Results**

127 3.1.Ascidian species and their status

128 During the study period, a total of 15 species of ascidians settled on the plates (Table 1).
129 Two were native, ten were introduced, and three were designated as cryptogenic, meaning
130 that there is insufficient information to assign them a native or introduced status (Carlton
131 1996).

132 The two native species *Phallusia mammillata* (Cuvier, 1815) and *Trididemnum cereum*
133 (Giard, 1872) have an Atlanto-Mediterranean distribution (Lafargue & Wahl 1987; Coll et
134 al. 2012). The latter was highly abundant on the oyster ropes.

135 The ten introduced species found on the plates are native to different regions of the world,

136 some were recently introduced and others have long been established in the
137 Mediterranean.

138 *Apolidium accarensense* (Millar, 1953) was recently introduced in the Mediterranean, where it is
139 found on Spanish and Italian shores (López-Legentil et al. 2015). It was described from W
140 Africa and Cape Verde Islands and has also been found in S Brazil (Rocha et al. 2005).
141 The species seems to be undergoing an expansion of its distribution range.

142 *Asciidiella aspersa* (Müller, 1776) was described in the NE Atlantic Ocean and is common
143 in Atlantic European shores from where it has spread to other areas such as the
144 Mediterranean, NW and SW Atlantic, South Africa, India, and N and S Pacific (Locke 2009;
145 Callahan et al. 2010; Tatián et al. 2010; Nishikawa et al. 2014), where it is found in
146 harbours and artificial environments (Nishikawa et al. 2014).

147 *Ciona robusta* Hoshino & Tokioka, 1967 is an introduced species described in Japan and
148 formerly identified as *C. intestinalis* (Linnaeus, 1767). The cosmopolitan taxon *Ciona*
149 *intestinalis* was recently shown to comprise several cryptic species, of which the most
150 widespread are the so-called *Ciona intestinalis* type A and type B (Caputi et al. 2007; Zahn
151 et al. 2010). Recent work (Brunetti et al. 2015; Pennati et al. 2015) has shown that *C.*
152 *intestinalis* type A is in fact *C. robusta*, a species present in both sides of the Pacific, the
153 Indian Ocean, the English Channel, and the Mediterranean. Recent genetic data
154 (Bouchemousse et al. 2016a) support the introduced status of *C. robusta* in Europe.

155 *Clavelina lepadiformis* (Müller, 1776) was described in the NE Atlantic and has expanded
156 to other areas (Azores, Madeira, South Africa, NW Atlantic and NW Pacific (Wirtz 1998;
157 Monniot et al. 2001; Reinhardt et al. 2010; Pyo et al. 2012). In the Atlanto-Mediterranean
158 region, genetic studies have shown that the form inhabiting marinas and artificial
159 substrates in the Mediterranean is an Atlantic lineage (likely a cryptic species) introduced
160 into the Mediterranean (Tarjuelo et al. 2001; Turon et al. 2003).

161 *Clavelina oblonga* Herdman, 1880 is native to the Caribbean area and was introduced in
162 Brazil and NE Atlantic (Rocha et al. 2012). It has been recently reported from the
163 Mediterranean (Ordóñez et al. 2016). *Clavelina phlegraea*, described in S Italy and found
164 also in Corsica (Salfi 1929; Monniot et al. 1986) is in fact a synonym of *C. oblonga* and
165 thus the introduction into the Mediterranean is relatively old (Ordóñez et al. 2016).

166 *Didemnum vexillum* Kott, 2002 is one of the potentially most harmful ascidian invaders
167 worldwide. It covers extensively artificial substrates and shellfish facilities, but it can also
168 proliferate in natural habitats impacting local communities (Valentine et al. 2007; Mercer et
169 al. 2009). This species is considered native to the NW Pacific but has become established
170 in temperate and cold regions worldwide (Lambert 2009; Stefaniak et al. 2012; Ordóñez et
171 al. 2015). In the Mediterranean it was recently reported from the Venice Lagoon
172 (Tagliapietra et al. 2012) and in the Ebro Delta area (Ordóñez et al. 2015).

173 *Diplosoma listerianum* (Milne Edwards, 1841) is now known to comprise a complex of
174 cryptic species distributed worldwide (Locke 2009). The clade found in the Mediterranean,
175 Clade A in Pérez-Portela et al. (2013), is native to the Atlantic and has been introduced in
176 many areas of the world, including the Mediterranean, where it is abundant in artificial and
177 altered environments.

178 *Microcosmus squamiger* Michaelsen, 1927 is a well-known worldwide invader, native to
179 Australia and distributed in temperate waters in the Indian, Pacific, and Atlantic Oceans
180 (Rius et al. 2008, 2012). In the Mediterranean, it is known since 1963 but has often been
181 confounded with *M. exasperatus* (Turon et al. 2007). It thrives in artificial habitats, but it
182 can also colonise adjacent natural substrates (Turon et al. 2007; Ordóñez et al. 2013a).

183 *Polyandrocarpa zorritensis* (Van Name, 1931) was described in Perú (Van Name 1931)
184 and later found in Brazil (Millar 1958). It is introduced in the Mediterranean, having been
185 recorded in Italy (Brunetti 1978; Brunetti & Mastrototaro 2004) and Spain (Turon & Perera
186 1988; López-Legentil et al. 2015), always in enclosed environments.

187 *Styela plicata* (Lesueur, 1823) is a cosmopolitan species, considered native to the NW
188 Pacific (Barros et al. 2009), that has been introduced in tropical and temperate waters
189 worldwide (Pineda et al. 2011). It is an old introduction in the Mediterranean.

190 The three cryptogenic species were *Botrylloides leachii* (Savigny, 1816), *Botryllus*
191 *schlosseri* (Pallas, 1776) and *Ecteinascidia turbinata* Herdman, 1880. *Botrylloides leachii*
192 was described in the Mediterranean and is found in all European shores, and in South
193 Africa, Australia and the Western Pacific (Locke 2009). It must be noted, however, that
194 confusion between *B. leachii* and other *Botrylloides* species has often occurred (Bishop et
195 al 2015). While a Mediterranean origin of this species has been suggested (Berrill 1950),
196 other authors consider that it can be an old introduction from the Pacific Ocean, the centre
197 of botryllid diversity (Carlton 2005).

198 The golden star tunicate *Botryllus schlosseri* is distributed worldwide; it has a marked
199 polymorphism in chromatic patterns and colony shapes, and indeed several colour
200 varieties were observed on our plates. It was recently shown that *B. schlosseri* is a
201 complex of five genetically differentiated clades (López-Legentil et al. 2006; Bock et al.
202 2012), all of them present in the Mediterranean. It is still unclear which is the native area of
203 the most invasive Clade A (Lejeune et al. 2011; Nydam et al. 2017; Reem et al. 2017).

204 *Ecteinascidia turbinata* is also cryptogenic in the Mediterranean (Maciver et al. 2016). It
205 has an amphi-Atlantic distribution in tropical and subtropical habitats with high genetic
206 homogeneity (López-Legentil & Turon 2007); in the W Mediterranean it is found on artificial
207 and estuarine/lagoonal habitats in the Balearic Islands and in the South of Spain. This
208 report represents a northward expansion of this species, likely linked to warming
209 temperatures. Even if it was not an introduced species, given its capacity to reach high
210 densities in favourable habitats, it constitutes a potential threat for aquaculture activities in
211 the studied area.

212

213 3.2. Occurrence and cover

214 The occurrences of the ascidians (total number of plates in which a given species was
215 present) differed in frequency, season and depth (Table 1). Six species occurred less than
216 50 times, three appeared from 50 to 99 times and another 6 were very common occurring
217 at least 100 times (Table 1). Most species showed a slightly higher occurrence during
218 colder seasons, but at all seasons there were at least 7 ascidian species present. Most (10
219 of the 15) were mainly found at 0.2 m, while four were more frequent at 2 m depth
220 (*Ascidia aspersa*, *Ciona robusta*, *Clavelina lepadiformis*, *Phallusia mammillata*). Only
221 *Ecteinascidia turbinata* showed preference for 1-2 m depth (Table 1). Considering the
222 maximum number of plates in which a species appeared in a given month (Table 1), seven
223 species were highly ubiquitous, appearing in 30 or more plates (out of 45): *T. cereum*, *A.*
224 *accarense*, *C. lepadiformis*, *D. listerianum*, *S. plicata*, *B. schlosseri*, *E. turbinata*. When
225 considering the maximum number of months in which a species was present in a given
226 plate as an estimate of persistence (Table 1), this value was highest (more than 10 mo out
227 of 19) for *T. cereum*, *C. lepadiformis*, *D. listerianum*, *S. plicata* and *B. schlosseri*. The most
228 ubiquitous species tended also to be the most persistent over time (Spearman correlation
229 coefficient: 0.721, p=0.002).

230 For the study of cover over time, we selected the species with at least 100 occurrences,
231 with three exceptions: *Didemnum vexillum* was included even if it appeared late in the
232 study (23 occurrences in summer and fall 2016) because it is abundant in the nearby
233 oyster ropes and is a well-known nuisance in these cultures (Ordóñez et al. 2015);
234 *Diplosoma listerianum* and *Botryllus schlosseri*, on the other hand, were excluded in spite
235 of their abundance due to the difficulty in delimiting the colonies' outlines in the pictures.
236 Therefore, the species selected for the cover study were the native species *Trididemnum*
237 *cereum*, the introduced species *Clavelina lepadiformis*, *Clavelina oblonga*, *Didemnum*
238 *vexillum*, and *Styela plicata*, and the cryptogenic species *Ecteinascidia turbinata*

239 Some species showed higher growth at a particular depth and this preference was usually
240 maintained for all the seasons of the study (Table 2). During fall 2015, the effect of
241 placement date of the ropes was more pronounced, but this effect tended to diminish over
242 time. Considering the whole studied period, only two species, *Ecteinascidia turbinata* and
243 *Styela plicata*, showed a significant difference in cover between dates of placement, with
244 higher overall growth on the plates placed earlier in the study (Table 2). *Clavelina*
245 *lepadiformis* and *S. plicata* were the most abundant species on the plates (Figure 2) and
246 were present during the entire study period (Table 1). Their mean cover was about 20%
247 (Figure 2), with a maximum peak of 80%. *S. plicata* had an overall higher growth on plates
248 placed at 0.2m depth and also on plates placed during May, June and July (Table 2). *C.*
249 *lepadiformis* showed a significant trend of higher growth at 1 and 2m depth since the
250 beginning of the study, with a tendency to grow more at 2m. Although it did not show
251 significant differences of cover between dates of placement, it did tend to grow better on
252 ropes placed on May and June (Table 2; Figure 2).

253 *Ecteinascidia turbinata* was present during most of the study period, but showed a marked
254 seasonality, appearing from end of summer to fall. The cover during the second year was
255 much higher than the first year (Figure 2), reaching mean values around 2% (the
256 maximum cover recorded in a single plate was 15%). This species showed significant
257 differences in cover for both depth and date of placement of the rope. On fall 2015, it grew
258 mostly at 2m depth, but considering all the months of the study it showed a preference for
259 1m depth (Table 2; Figure 2). Regarding the date of placement, this species showed
260 significantly higher growth on those ropes placed on May and June.

261 The other three species, *Clavelina oblonga*, *Didemnum vexillum* and *Trididemnum cereum*
262 showed no significant preferences for either a specific depth nor date of placement of the
263 rope when considering the whole study period (Table 2). Among these three species, the
264 most abundant was *T. cereum*, which showed mean cover of up to ca. 7% (maximum
265 cover recorded in a single plate was 25%) and a marked seasonality, appearing almost
266 exclusively during winter and spring 2016 (Figure 2). *C. oblonga* was the less abundant in
267 terms of cover (Figure 2), as the maximum cover on a single plate was less than 1%. It
268 also showed a marked seasonality, with regression during the summer months and a peak
269 in fall. Finally, *D. vexillum*, although it was present during most of the time on the nearby
270 bivalve culture ropes, only appeared on the plates during summer 2016 for the first time,
271 with a maximum peak of 5% of cover during fall 2016. It showed a preference for shallower
272 depths although the differences in cover with depth were not significant when considering
273 the whole study period (Table 2, Figure 2).

274 The environmental abiotic parameters (i.e., temperature, salinity, oxygen) were measured
275 at 1 metre depth, and the concentration of Chlorophyll *a* was measured from the integrated
276 water column sample (Figure 3). The temperature ranged between 5.43°C and 29.72°C,
277 with an average (\pm SE) of 18.34°C (\pm 0.62). This wide range is due to the shallowness of the
278 aquaculture area. The salinity ranged between 23.88 and 37.93, influenced by the Ebro
279 River inputs, with an average (\pm SE) of 33.5 (\pm 0.28). The minimum salinity values were

280 obtained in November 2015. The percentage of oxygen ranged between 54.5% and
281 136.9%, with an average (\pm SE) of 87.12% (\pm 1.15). Its values were lowest in October of
282 both years. Finally, the concentration of Chlorophyll *a* ranged from 0.39 to 9.12 $\mu\text{g}\cdot\text{L}^{-1}$,
283 with an average (\pm SE) of 1.80 $\mu\text{g}\cdot\text{L}^{-1}$ (\pm 0.13). Peaks in Chlorophyll *a* were detected in
284 summer months, but also in February 2016.

285 The results of the cross-correlation analyses showed that three species had a significant
286 correlation of cover with water temperature in previous months (Table 3). *Clavelina*
287 *oblonga* and *Ecteinascidia turbinata* had a positive correlation, indicating that their growth
288 was enhanced by warmer temperatures the months before. *Trididemnum cereum* showed
289 a negative correlation, pointing to higher growth after the cold season. The other variables
290 only showed significant correlations with cover values in a few instances: a negative
291 correlation for *C. oblonga* and a positive correlation for *T. cereum* with salinity of previous
292 months; a negative correlation for *C. oblonga* with oxygen of the present and previous
293 months; and a positive correlation for *E. turbinata* with Chlorophyll *a* of present and
294 previous months (Table 3).

295

296

297 4. Discussion

298 A high diversity of ascidians was detected on settlement plates deployed in the
299 aquaculture facility studied. Fifteen species were identified; ten could be assigned an
300 introduced status, while another three were cryptogenic. This finding confirms the
301 important role of aquaculture activities as vectors for non-indigenous species (Rius et al.
302 2011; Fitritge et al. 2012).

303 The ascidian fauna of the same bay was examined almost three decades ago (Turon &
304 Perera 1988; Perera et al. 1990). The same number of ascidian species (15) were
305 reported then, but with some significant differences. Five species reported here were not
306 mentioned in the previous studies: *Aplidium accareense*, *Clavelina oblonga*, *Didemnum*
307 *vexillum*, *Diplosoma listerianum* and *Ecteinascidia turbinata*. With the possible exception of
308 *D. listerianum* (well-known in W Mediterranean from long ago), the other four are likely
309 new introductions, reflecting a worrisome trend of increasing numbers of non-native
310 ascidians (Zenetos et al. 2017). Another five species detected in previous works were not
311 found in this study: *Aplidium densum*, *Lissoclinum perforatum*, *Perophora viridis*,
312 *Polycarpa pomaria* and *Pyura dura*. It is difficult to know whether these species have
313 disappeared or whether they are just less abundant at this aquaculture facility and
314 escaped detection in our study. The remaining species reported here were already present
315 in the late 1980's (note that *Ciona robusta* and *Microcosmus squamiger* were formerly
316 identified under the names *C. intestinalis* and *M. exasperatus*, respectively).

317 Settlement plates have been the method of choice in studies of invasive ascidians (see
318 review in Cordell et al. 2013), both for descriptive (e.g., Marins et al. 2010; Tracy & Reynolds
319 2014; Valentine et al. 2016) and experimental approaches (e.g., Simkanin et al. 2013,
320 2017; Kremer & Rocha 2016). However, PVC plates may not be the best surrogate for the
321 available substrate in the area, which is mostly the bivalve surfaces, and some biases in
322 species composition and abundance are expected. Indeed, the composition of species
323 found on the plates during some months of the monitoring was quite different from that
324 observed on the nearby bivalves on culture ropes. Such differences in settlement between
325 substrates may be due to different causes. The nature of the plate material and its
326 roughness can determine larval settlement preferences (Chase et al. 2016). In addition,
327 newly placed plates do not have the biofilm, irregularities and potential hiding places that

328 develop over time as fouling progresses and that are crucial for the successful settlement
329 of some species. After one year submerged, the plates were covered with barnacles,
330 bryozoans and ascidians, creating a complex substrate, like that created by oysters and
331 mussels on the bivalve culture ropes. While for some ascidians the availability of bare
332 space, free from competitors, is necessary for recruitment and survival, others require the
333 increased surface complexity afforded by established fouling species (Simkanin et al.
334 2017). Two species, *Clavelina oblonga* and *Didemnum vexillum*, showed very low cover on
335 the plates in contrast with the extremely high cover found on bivalve cultures. *C. oblonga*
336 is very abundant in the southern Bay of the Ebro Delta (Alfacs Bay), where it is a major
337 pest (Ordóñez et al. 2016). During the study, it was present on the bivalve culture ropes
338 from Fangar Bay with less cover than in Alfacs Bay but still much higher than on our PVC
339 plates. Similarly, *D. vexillum* did not grow during the first year on the plates, in contrast
340 with the abundance and size of the colonies on bivalve culture ropes during the same
341 period, and in spite of having been initially deployed during the reproductive period of the
342 ascidian in the area (Ordóñez et al. 2015). Thus, for the purpose of monitoring activities for
343 ascidian detection and abundance estimates the best strategy is to use both clean and
344 colonized experimental surfaces.

345 The interplay of settlement dynamics and environmental changes determines the
346 outcomes of competitive interactions among ascidians on artificial substrates
347 (Bouchemousse et al. 2016b). Shifts in dominant species over the seasons and early biotic
348 interactions have important implications for the coexistence of species and the diversity of
349 fouling communities (Dijkstra & Harris 2009; Ordóñez et al. 2013b). We have detected a
350 strong seasonality in most of the species, so that during some periods they are reduced or
351 absent. This generates an alternance in dominant species and provides opportunities for
352 settlement on previously occupied surfaces, thus contributing to successful coexistence of
353 fouling organisms. The species' dominance was also different from one year to the other.
354 For instance, *Styela plicata* presented a markedly higher cover during the summer of 2015,
355 shortly after initial deployment, than in the same period of 2016, indicating that it is an
356 opportunistic species. Once a species is successfully settled, it can provide a substrate for
357 other species, so interactions between species can differ depending on the first settlers.
358 The massive presence of *S. plicata* at the beginning of the study would probably enhance
359 the settlement of some species and inhibit others. For instance, *Diplosoma listerianum*
360 was observed to grow frequently on *S. plicata* and, similarly its frequency of occurrence
361 during summer 2016 was much lower than that in summer 2015. Conversely,
362 *Ecteinascidia turbinata*, whose seasonality overlaps partially with that of *S. plicata*, showed
363 higher cover during end of summer and fall 2016 than in the same period on 2015. These
364 are correlations but the extent to which one species has had a direct influence on another
365 would require specific experimental studies.

366 Aside from the importance of the substrate and the interspecific interactions, the date of
367 placement of the ropes is another important factor. The coupling of reproductive cycles
368 with the availability of substrate is key to the establishment of species. We have observed
369 that the date of deployment of the ropes had a clear effect on the cover of most species
370 during the initial seasons, and tended to diminish with time. However, for some species,
371 the effect of initial date was still significant at the end of the study. Species such as
372 *Clavelina lepadiformis*, that reproduces in winter-spring (De Caralt et al. 2002),
373 *Ecteinascidia turbinata*, with reproduction in spring-summer (Carballo 2000), or *Styela*
374 *plicata*, with continuous breeding but with peaks in spring (Pineda et al. 2013), tend to
375 develop more on ropes placed during May and June, and this effect can be long-lasting
376 and still appreciable at the end of the study after several cycles of regression-
377 reappearance (cf. *C. lepadiformis* and *E. turbinata*, Figure 2).

378 The Ebro Delta is both a major center of bivalve production and a hotspot for invasive
379 species. Foulers such as ascidians are a nuisance of concern as they decrease bivalve
380 productivity (Daigle & Herbinger 2009). The establishment of a monitoring programme in
381 aquaculture facilities is of crucial importance. Although we could measure cover only for
382 some of the species, we had data on occurrences for all of them. We could detect that
383 some species were ubiquitous and persistent, occurring in many plates over many months,
384 thus deserving the highest concern. We found that occurrence rates and cover rates are
385 similarly informative. In most of the species, the peak in occurrence frequency took place
386 during the same period and at the same depth as the peak of cover, with some exceptions.
387 For instance, *Styela plicata* showed, during summer 2015, a very high cover but a low
388 occurrence (Fig. 2, Table 1). This difference may be due to the depth preference, as in
389 summer 2015 almost all the specimens of this species were concentrated in plates at 0.2
390 m depth. However, occurrence rates seem to be a good indicator of species abundance.
391 This suggests that a simple follow-up of occurrence of species in replicate plates, which is
392 much faster than analysing cover, would be sufficient for monitoring purposes, providing
393 an adequate picture of the dynamics of ascidian populations on plates.

394 Continued surveillance over time is the best way to detect new introduced species as soon
395 as possible, which is a pre-requisite for successful mitigation measures. It also provides
396 information about settlement preferences of key species, which can help minimise fouling.
397 Our study lasted for 20 months, slightly longer than the time required to grow oysters to a
398 commercial size (ca. 18 months). A recent study suggested that restricting the immersion
399 of spat to two periods, summer and end of autumn, could minimise mortality by the ostreid
400 herpesvirus microvar (Carrasco et al. 2017). The first period may not be advisable if the
401 objective is to minimise fouling. In this study, we detected for some of the species that
402 plates immersed earlier in the study (spring-early summer) had higher cover over the
403 whole study period, suggesting that avoidance of seeding during these months may
404 mitigate ascidian cover later in oyster development. Spring is the most common breeding
405 season for invertebrates in general in the Mediterranean (Coma et al. 2000), suggesting
406 that oysters placed in early summer would receive the strongest load of epibionts. In
407 addition, most ascidians showed a marked preference for growing at shallower depths, so
408 placing the bivalves below 1 m depth could substantially reduce fouling on them.
409 Whenever possible, husbandry practices focusing on the dates and depths of spat
410 immersion should be implemented, based on information from biomonitoring programs, to
411 reduce biofouling load over bivalve cultures.

412

413

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Table 1. TOTAL: number of occurrences for each species on all plates for the entire period of study. MAX: Maximum number of plates occupied in a single observation. PERS: Maximum number of months the species persisted in a given plate during the study (persistence). Frequencies of occurrence by season (combining the three months of observations of each season) and depth are also indicated. The highest percentages by season and depth for a given species are in bold. N=45 (15 ropes x 3 plates) except in Summer '15 when N=36 (the three ropes immersed in September were not used).

Status	Species	TOTAL	MAX	PERS	Season						Depth		
					Summer '15	Fall '15	Winter '16	Spring '16	Summer '16	Fall '16	0.2 m	1 m	2 m
Native	<i>Phallusia mammillata</i>	17	5	5	0.0%	0.0%	23.5%	52.9%	0.0%	23.5%	0.0%	41.2%	58.8%
	<i>Trididemnum cereum</i>	212	42	11	0.0%	0.0%	24.1%	41.5%	14.6%	19.8%	43.4%	34.0%	22.6%
Introduced	<i>Aplidium accareense</i>	71	37	4	8.5%	0.0%	1.4%	5.6%	0.0%	84.5%	46.5%	36.6%	16.9%
	<i>Asciidiella aspersa</i>	11	3	3	0.0%	0.0%	45.5%	27.3%	0.0%	27.3%	0.0%	36.4%	63.6%
	<i>Ciona robusta</i>	6	3	2	0.0%	0.0%	83.3%	16.7%	0.0%	0.0%	0.0%	0.0%	100.0%
	<i>Clavelina lepadiformis</i>	357	42	15	8.1%	7.6%	19.9%	29.1%	7.8%	27.5%	12.0%	41.5%	46.5%
	<i>Clavelina oblonga</i>	100	22	6	3%	47.5%	10.1%	3.0%	8.1%	28.3%	43.4%	30.3%	26.3%
	<i>Didemnum vexillum</i>	23	7	5	0.0%	0.0%	0.0%	0.0%	30.4%	69.6%	82.6%	8.7%	8.7%
	<i>Diplosoma listerianum</i>	189	31	13	37.0%	20.1%	25.4%	14.8%	0.5%	2.1%	59.3%	20.1%	20.6%
	<i>Microcosmus squamiger</i>	45	10	5	0.0%	0.0%	2.2%	11.1%	40.0%	46.7%	46.7%	35.6%	17.8%
	<i>Polyandrocarpa zorritensis</i>	2	1	1	0.0%	0.0%	100.0%	0.0%	0.0%	0.0%	100.0%	0.0%	0.0%
	<i>Styela plicata</i>	330	30	19	10.6%	8.2%	24.2%	23.9%	15.2%	17.9%	56.4%	29.4%	14.2%
Cryptogenic	<i>Botrylloides leachii</i>	72	13	7	5.6%	9.7%	12.5%	38.9%	22.2%	11.1%	75.0%	18.1%	6.9%
	<i>Botryllus schlosseri</i>	323	30	14	37.2%	17.3%	21.1%	18.9%	0.3%	5.3%	48.6%	20.7%	30.7%
	<i>Ecteinascidia turbinata</i>	125	32	7	7.2%	17.6%	0.8%	0.0%	41.6%	32.8%	15.2%	43.2%	41.6%

Table 2. Summary of PERMANOVA analyses of the percent cover for each season of study (Fall '15 to Fall '16) and for the whole period (TOTAL). "NA" indicates that there were not enough observations in the given season to carry out the analysis. Significant results are highlighted in bold. For the TOTAL results, when the date of placement or the depth factors were significant, the results of pairwise tests between and dates (M: May, J: June, Jl: July, A: August, S: September) and depths (0.2, 1 and 2 m) are presented.

Status	Species	Factor	df	Fall'15	Winter'16	Spring'16	Summer'16	Fall'16	TOTAL	Pairwise comparisons
				Pseudo-F	P	Pseudo-F	P	Pseudo-F	P	
Native	<i>Trididemnum cereum</i>	Date	4		1.7824 0.193	2.063 0.137	4.701 0.028	0.862 0.616	1.870 0.176	
		Depth	2		3.2613 0.052	3.553 0.054	48.537 0.001	2.499 0.094	3.115 0.063	
		Rope(Date)	1	NA	1.8174 0.109	1.502 0.202	1.233 0.355	0.899 0.670	1.806 0.103	
		DatexDepth	0 8		1.3145 0.299	1.918 0.105	5.072 0.001	0.649 0.794	1.941 0.111	
Introduced	<i>Clavelina lepadiformis</i>	Date	4	5.630 0.003	2.404 0.085	2.450 0.093	0.714 0.595	2.402 0.089	2.711 0.074	(1, 2 > 0.2)
		Depth	2	16.211 0.001	7.947 0.001	6.931 0.005	4.291 0.031	7.298 0.005	7.625 0.004	
		Rope(Date)	1	1.813 0.101	0.997 0.502	0.806 0.625	0.693 0.732	0.930 0.566	0.800 0.654	
		DatexDepth	0 8	8.857 0.001	2.163 0.070	1.667 0.168	1.703 0.148	1.963 0.105	1.897 0.117	
	<i>Clavelina oblonga</i>	Date	4	3.695 0.061	1.751 0.157		0.205 0.914	1.012 0.450	0.974 0.451	
		Depth	2	1.131 0.358	1.382 0.296		2.504 0.118	3.988 0.037	0.732 0.529	
		Rope(Date)	1	0.627 0.809	3.041 0.019	NA	1.000 0.474	1.869 0.044	1.170 0.344	
		DatexDepth	0 8	1.102 0.417	0.981 0.478		1.767 0.137	1.218 0.320	1.129 0.376	
	<i>Didemnum vexillum</i>	Date	4				1.459 0.408	1.867 0.104	1.782 0.119	
		Depth	2				4.047 0.029	1.614 0.226	1.790 0.194	
		Rope(Date)	1	NA	NA	NA	1.000 0.527	0.658 0.797	0.679 0.795	
		DatexDepth	0 8				1.459 0.212	0.467 0.899	0.509 0.881	
	<i>Styela plicata</i>	Date	4	19.501 0.001	4.312 0.016	1.627 0.207	3.077 0.059	1.253 0.338	5.042 0.029	M,J,Jl > A,S (0.2 > 1 > 2)
		Depth	2	36.744 0.001	14.739 0.001	8.835 0.005	11.433 0.001	25.680 0.001	58.332 0.001	
		Rope(Date)	1	0.892 0.657	1.047 0.439	1.583 0.176	2.122 0.062	2.415 0.037	1.829 0.113	
		DatexDepth	0 8	16.451 0.001	4.699 0.001	1.320 0.283	3.930 0.006	2.390 0.055	4.460 0.004	
Cryptogenic	<i>Ecteinascidia turbinata</i>	Date	4	19.501 0.002			7.028 0.009	6.318 0.021	7.396 0.013	M,J > Jl,A,S (1 > 2 > 0.2)
		Depth	2	36.744 0.001			5.279 0.014	8.271 0.005	9.236 0.003	
		Rope(Date)	1	0.892 0.650	NA	NA	0.600 0.814	1.090 0.402	1.260 0.310	
		DatexDepth	0 8	16.451 0.001			0.481 0.882	2.211 0.058	1.888 0.099	

Table 3. Summary of cross-correlation tests between each environmental parameter and the percent cover of each species. The tests were run for the same (0) and previous months (-1, -2, -3). Correlation coefficients are indicated and significant values are in bold.

Status	Species	Month compared	Temperature	Salinity	Oxygen	Chlorophyll a
Native	<i>Trididemnum cereum</i>	-3	-0.619	0.427	-0.096	-0.321
		-2	-0.638	0.533	0.025	-0.111
		-1	-0.510	0.371	-0.020	-0.358
		0	-0.286	0.322	-0.060	-0.403
Introduced	<i>Clavelina lepadiformis</i>	-3	-0.275	0.431	-0.067	0.092
		-2	-0.328	0.392	-0.316	0.064
		-1	-0.416	0.267	-0.181	-0.154
		0	-0.350	0.046	0.013	-0.433
	<i>Clavelina oblonga</i>	-3	0.528	-0.302	0.086	0.313
		-2	0.265	-0.377	-0.311	0.221
		-1	-0.074	-0.612	-0.702	0.260
		0	-0.419	-0.408	-0.529	-0.039
	<i>Didemnum vexillum</i>	-3	0.316	0.029	0.017	0.407
		-2	0.156	-0.040	-0.421	0.279
		-1	-0.139	0.022	-0.068	0.279
		0	-0.283	-0.052	0.090	-0.132
<i>Styela plicata</i>	-3	-0.298	0.308	0.014	0.014	
	-2	-0.140	0.278	0.295	0.082	
	-1	-0.105	0.307	0.237	-0.088	
	0	-0.132	0.082	-0.096	-0.332	
Cryptogenic	<i>Ecteinascidia turbinata</i>	-3	0.410	-0.047	0.184	-0.020
		-2	0.506	0.060	0.087	0.308
		-1	0.403	-0.046	-0.167	0.513
		0	0.184	-0.090	-0.378	0.466

Figure captions

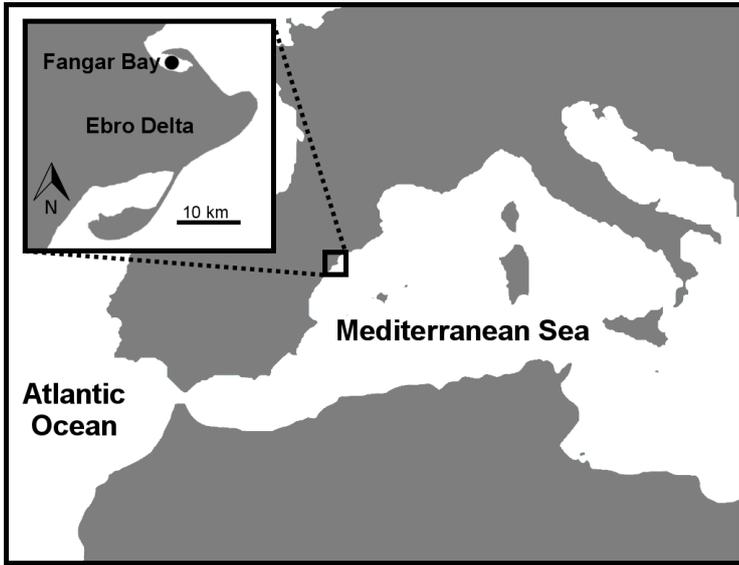


Figure 1.) Location of the study area, Fangar Bay.

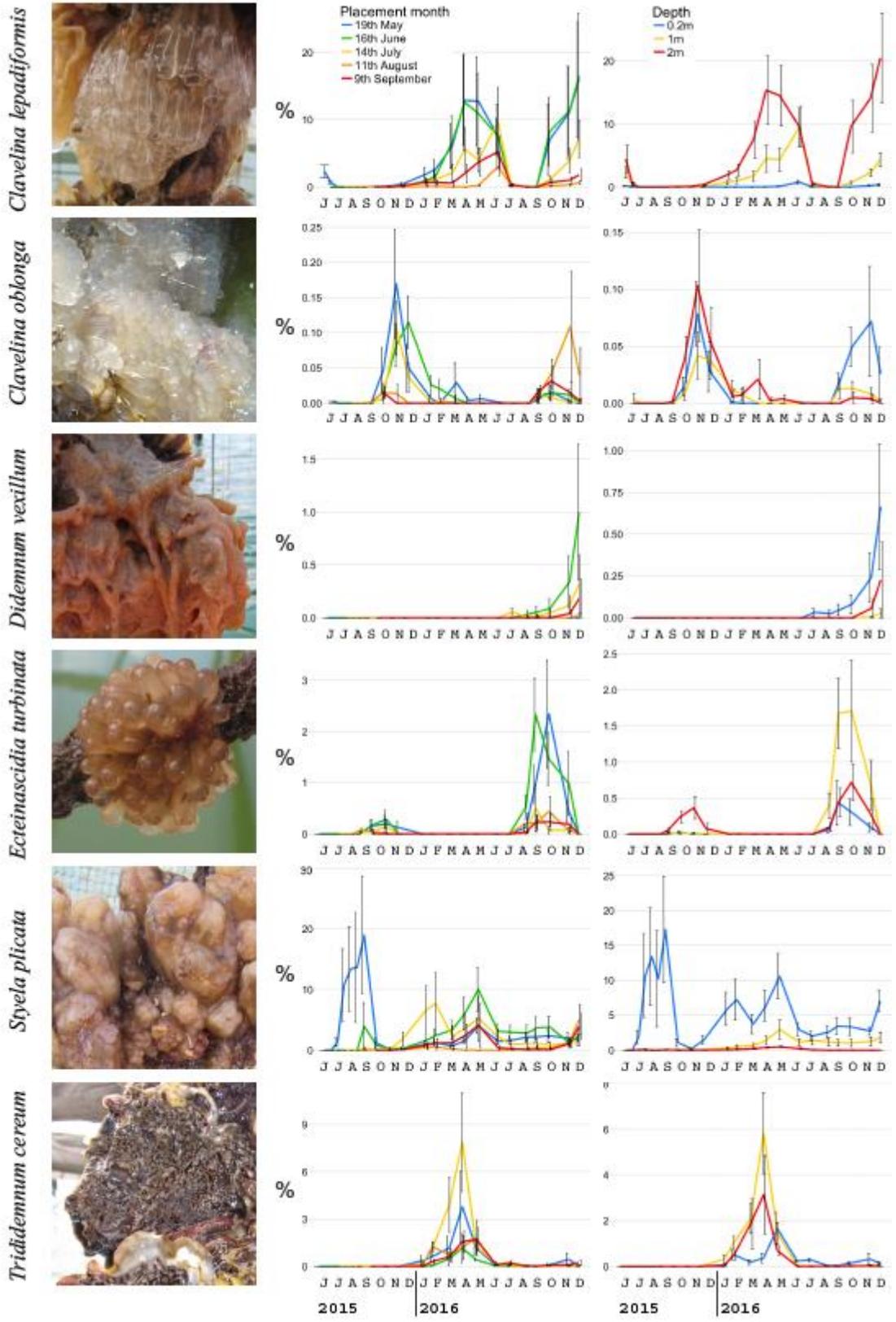


Figure 2. Percent cover over time for each selected species by date of placement and depth

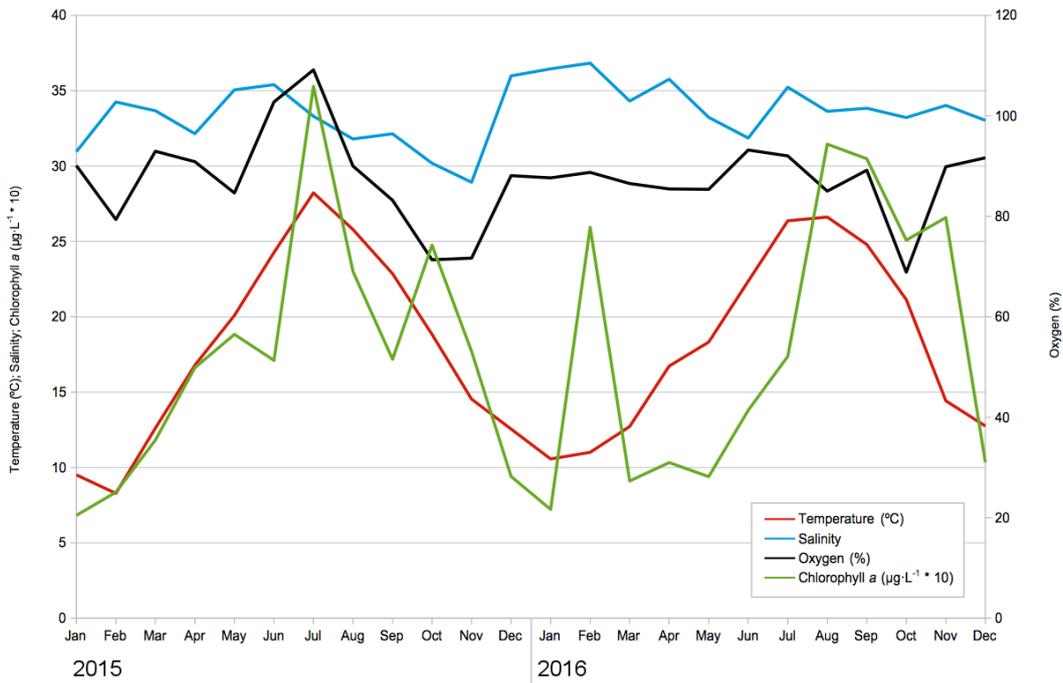


Figure 3. Temperature ($^{\circ}\text{C}$), salinity (psu) and oxygen (%) at 1 metre depth, and Chlorophyll a ($\mu\text{g L}^{-1} * 10$) from the integrated water column, over time. The values represented correspond to monthly means calculated from weekly measures.