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

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Abstract

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

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

1.Introduction

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

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

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

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

2.Material & Methods

The study site was located in Fangar Bay, at the northern side of the Ebro Delta in the NE Iberian Coast (40º46’27.43”N, 0º44’27.11”E, Figure 1). Fangar Bay has 9 km^2 of surface area with a muddy bottom up to 4.2 m depth. Bivalve rafts are used to grow the oyster *Crassostrea gigas* and the mussel *Mytilus galloprovincialis*. Each raft is supported by cement pilings and consists of a rectangular structure of wooden beams arranged in a grid, from which the bivalve ropes hang.

We used a raft located in the middle of the aquaculture facilities to hang a total of 15 ropes with settlement plates. All ropes were placed on the same side of the raft and were interspersed along its length. From May to September 2015, 3 ropes were placed each month and left in place until the end of the study. On each rope, 3 PVC plates pre-roughened with coarse sandpaper, 20x20 cm in size, were vertically attached with tiewraps, at 0.20, 1 and 2 m depth, respectively.

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

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

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

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

3.Results

3.1.Ascidian species and their status

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

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

The ten introduced species found on the plates are native to different regions of the world, some were recently introduced and others have long been established in the Mediterranean.

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

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

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

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

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

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

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

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

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

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

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

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

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

3.2.Occurrence and cover

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

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

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

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

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

The environmental abiotic parameters (i.e., temperature, salinity, oxygen) were measured at 1 metre depth, and the concentration of Chlorophyll *a* was measured from the integrated water column sample (Figure 3). The temperature ranged between 5.43ºC and 29.72ºC, with an average (±SE) of 18.34ºC (±0.62). This wide range is due to the shallowness of the aquaculture area. The salinity ranged between 23.88 and 37.93, influenced by the Ebro River inputs, with an average (±SE) of 33.5 (±0.28). The minimum salinity values were obtained in November 2015. The percentage of oxygen ranged between 54.5% and 136.9%, with an average (±SE) of 87.12% (±1.15). Its values were lowest in October of both years. Finally, the concentration of Chlorophyll *a* ranged from 0.39 to 9.12 µg·L-1, with an average (±SE) of 1.80 µg·L-1 (±0.13). Peaks in Chlorophyll *a* were detected in summer months, but also in February 2016.

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

4.Discussion

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

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

Settlement plates have been the method of choice in studies of invasive ascidians (see review in Cordell et al. 2013), both for descriptive (e.g., Marins et al. 2010; Tracy & Reyns 2014; Valentine et al. 2016) and experimental approaches (e.g., Simkanin et al. 2013, 2017; Kremer & Rocha 2016). However, PVC plates may not be the best surrogate for the available substrate in the area, which is mostly the bivalve surfaces, and some biases in species composition and abundance are expected. Indeed, the composition of species found on the plates during some months of the monitoring was quite different from that observed on the nearby bivalves on culture ropes. Such differences in settlement between substrates may be due to different causes. The nature of the plate material and its roughness can determine larval settlement preferences (Chase et al. 2016). In addition, newly placed plates do not have the biofilm, irregularities and potential hiding places that develop over time as fouling progresses and that are crucial for the successful settlement of some species. After one year submerged, the plates were covered with barnacles, bryozoans and ascidians, creating a complex substrate, like that created by oysters and mussels on the bivalve culture ropes. While for some ascidians the availability of bare space, free from competitors, is necessary for recruitment and survival, others require the increased surface complexity afforded by established fouling species (Simkanin et al. 2017). Two species, *Clavelina oblonga* and *Didemnum vexillum*, showed very low cover on the plates in contrast with the extremely high cover found on bivalve cultures. *C. oblonga* is very abundant in the southern Bay of the Ebro Delta (Alfacs Bay), where it is a major pest (Ordóñez et al. 2016). During the study, it was present on the bivalve culture ropes from Fangar Bay with less cover than in Alfacs Bay but still much higher than on our PVC plates. Similarly, *D. vexillum* did not grow during the first year on the plates, in contrast with the abundance and size of the colonies on bivalve culture ropes during the same period, and in spite of having been initially deployed during the reproductive period of the ascidian in the area (Ordóñez et al. 2015). Thus, for the purpose of monitoring activities for ascidian detection and abundance estimates the best strategy is to use both clean and colonized experimental surfaces.

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

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

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

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

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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	*Phallusia mammillata*	17	5	5	0.0%	0.0%	23.5%	**52.9%**	0.0%	23.5%	0.0%	41.2%	**58.8%**
	Trididemnum cereum	212	42	11	0.0%	0.0%	24.1%	**41.5%**	14.6%	19.8%	**43.4%**	34.0%	22.6%
Introduced	*Aplidium accarense*	71	37	4	8.5%	0.0%	1.4%	5.6%	0.0%	**84.5%**	**46.5%**	36.6%	16.9%
	Ascidiella aspersa	11	3	3	0.0%	0.0%	**45.5%**	27.3%	0.0%	27.3%	0.0%	36.4%	**63.6%**
	Ciona robusta	6	3	2	0.0%	0.0%	**83.3%**	16.7%	0.0%	0.0%	0.0%	0.0%	**100.0%**
	Clavelina lepadiformis	357	42	15	8.1%	7.6%	19.9%	**29.1%**	7.8%	27.5%	12.0%	41.5%	**46.5%**
	Clavelina oblonga	100	22	6	3%	**47.5%**	10.1%	3.0%	8.1%	28.3%	**43.4%**	30.3%	26.3%
	Didemnum vexillum	23	7	5	0.0%	0.0%	0.0%	0.0%	30.4%	**69.6%**	**82.6%**	8.7%	8.7%
	Diplosoma listerianum	189	31	13	**37.0%**	20.1%	25.4%	14.8%	0.5%	2.1%	**59.3%**	20.1%	20.6%
	Microcosmus squamiger	45	10	5	0.0%	0.0%	2.2%	11.1%	40.0%	**46.7%**	**46.7%**	35.6%	17.8%
	Polyandrocarpa zorritensis	2	1	1	0.0%	0.0%	**100.0%**	0.0%	0.0%	0.0%	**100.0%**	0.0%	0.0%
	Styela plicata	330	30	19	10.6%	8.2%	**24.2%**	23.9%	15.2%	17.9%	**56.4%**	29.4%	14.2%
Cryptogenic	*Botrylloides leachii*	72	13	7	5.6%	9.7%	12.5%	**38.9%**	22.2%	11.1%	**75.0%**	18.1%	6.9%
	Botryllus schlosseri	323	30	14	**37.2%**	17.3%	21.1%	18.9%	0.3%	5.3%	**48.6%**	20.7%	30.7%
	Ecteinascidia turbinata	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	Pseudo-F	P	Pseudo-F	P	Pseudo-F	P	
Native	*Trididemnum cereum*	Date	4	NA		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			1.8174	0.109	1.502	0.202	1.233	0.355	0.899	0.670	1.806	0.103	
		DatexDepth	0			1.3145	0.299	1.918	0.105	**5.072**	**0.001**	0.649	0.794	1.941	0.111	
			8													
Introduced	*Clavelina lepadiformis*	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	
		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**	(1, 2 > 0.2)
		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	**8.857**	**0.001**	2.163	0.070	1.667	0.168	1.703	0.148	1.963	0.105	1.897	0.117	
			8													
	Clavelina oblonga	Date	4	3.695	0.061	1.751	0.157	NA		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**			1.000	0.474	**1.869**	**0.044**	1.170	0.344	
		DatexDepth	0	1.102	0.417	0.981	0.478			1.767	0.137	1.218	0.320	1.129	0.376	
			8													
	Didemnum vexillum	Date	4	NA		NA		NA		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							1.000	0.527	0.658	0.797	0.679	0.795	
		DatexDepth	0							1.459	0.212	0.467	0.899	0.509	0.881	
			8													
	Styela plicata	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
		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**	(0.2 > 1 > 2)
		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	**16.451**	**0.001**	**4.699**	**0.001**	1.320	0.283	**3.930**	**0.006**	2.390	0.055	**4.460**	**0.004**	
			8													
Cryptogenic	*Ecteinascidia turbinata*	Date	4	**19.501**	**0.002**	NA		NA		**7.028**	**0.009**	**6.318**	**0.021**	**7.396**	**0.013**	M,J > Jl,A,S
		Depth	2	**36.744**	**0.001**					**5.279**	**0.014**	**8.271**	**0.005**	**9.236**	**0.003**	(1 > 2 > 0.2)
		Rope(Date)	1	0.892	0.650					0.600	0.814	1.090	0.402	1.260	0.310	
		DatexDepth	0	**16.451**	**0.001**					0.481	0.882	2.211	0.058	1.888	0.099	
			8													

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.

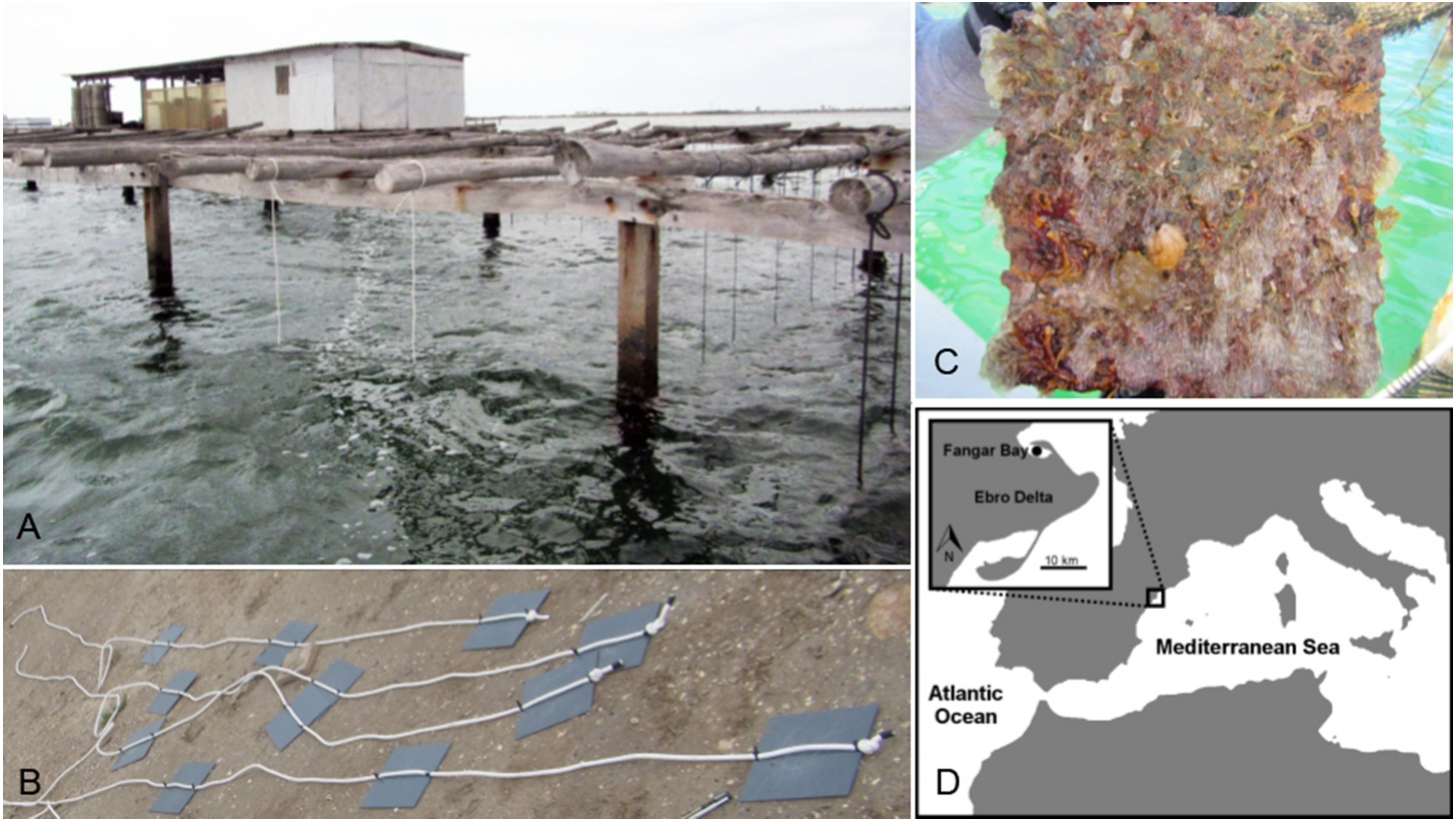
Status	Species	Month compared	Temperature	Salinity	Oxygen	Chlorophyll *a*
Native	*Trididemnum cereum*	-3	**-0.619**	0.427	-0.096	-0.321
		-2	**-0.638**	**0.533**	0.025	-0.111
		-1	**-0.510**	0.371	-0.020	-0.358
		0	-0.286	0.322	-0.060	-0.403
Introduced	*Clavelina lepadiformis*	-3	-0.275	0.431	-0.067	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
	Clavelina oblonga	-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
	Didemnum vexillum	-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
	Styela plicata	-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	*Ecteinascidia turbinata*	-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**

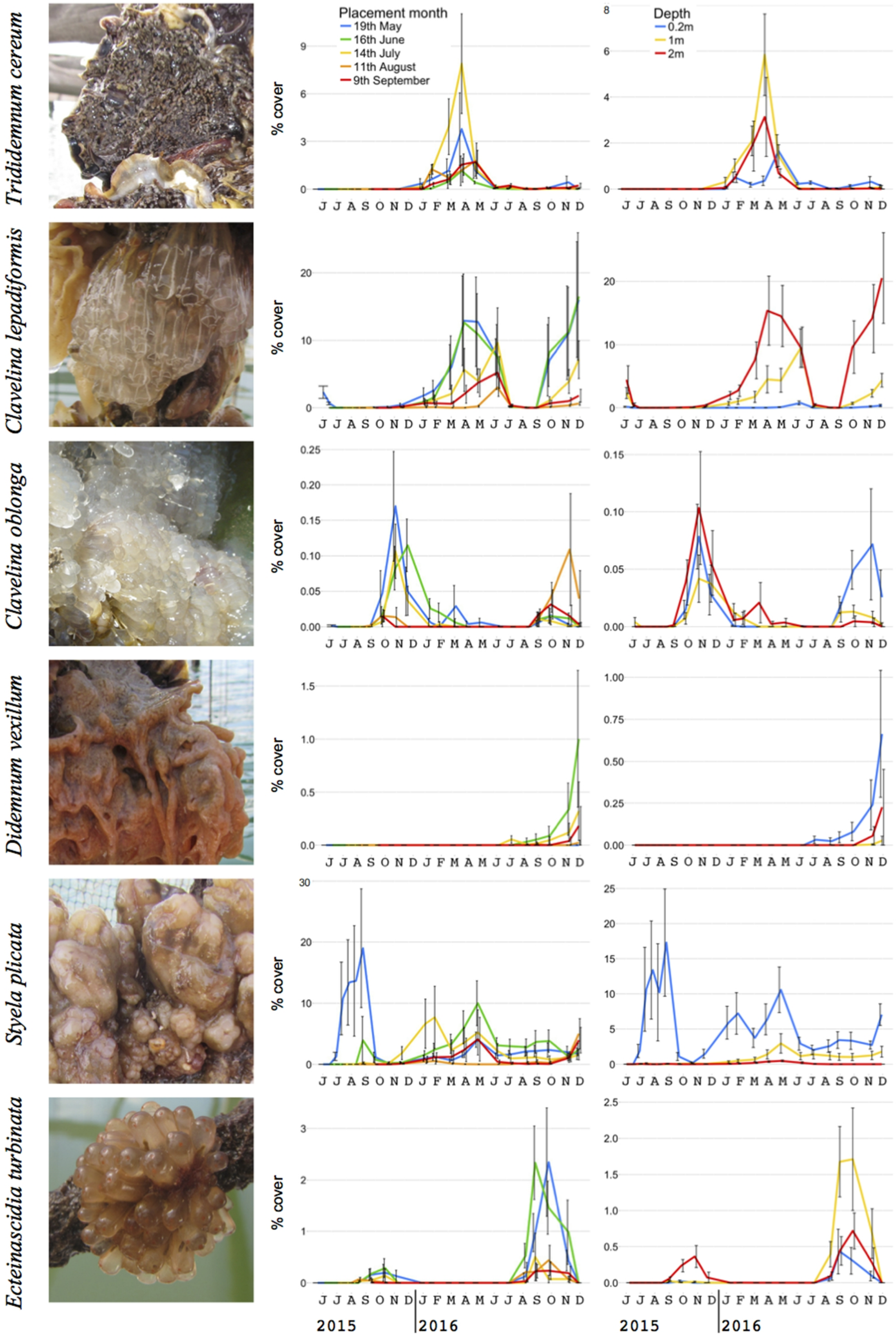
Figure captions

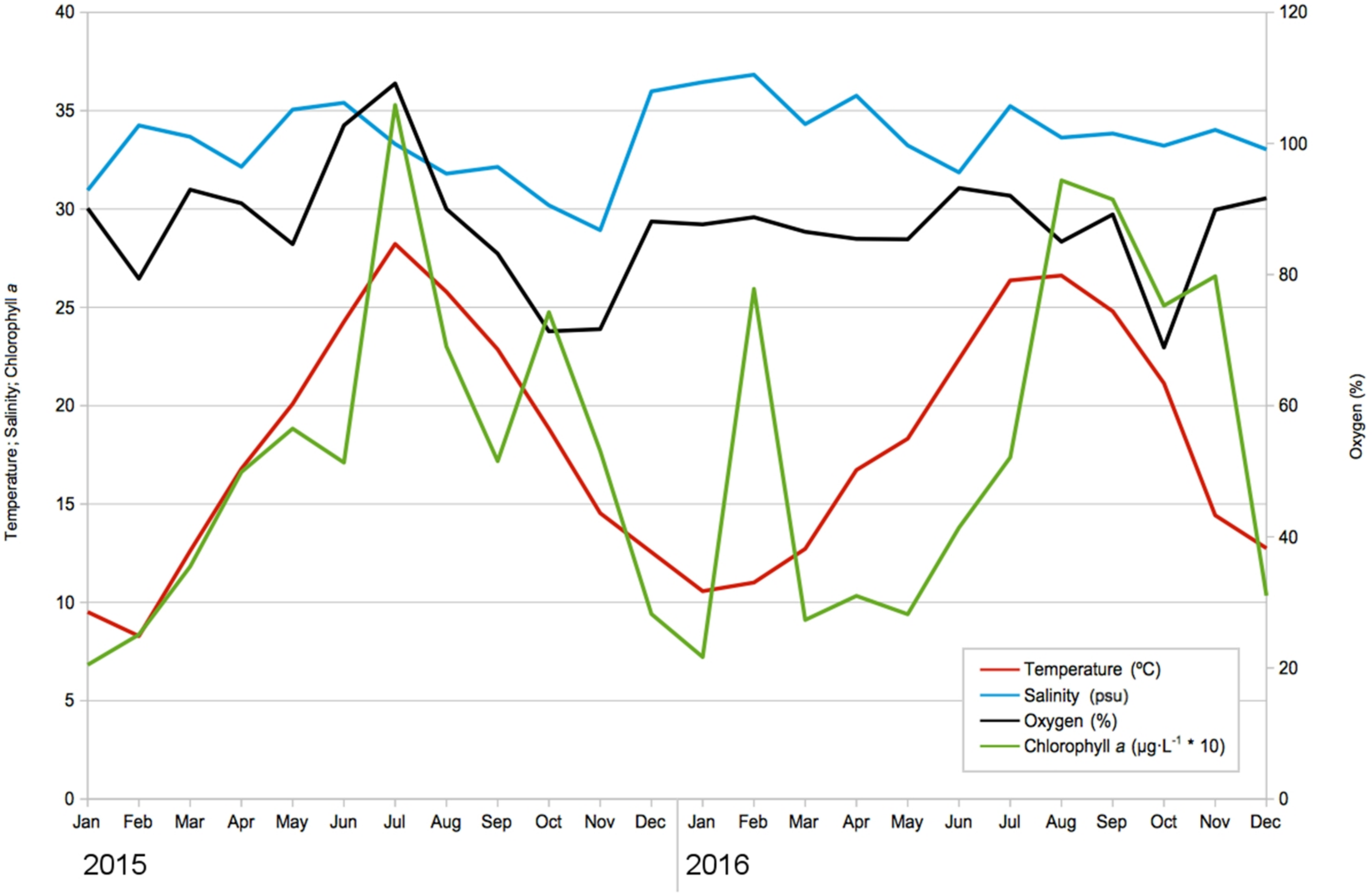
Figure 1. A) Raft used to hang the ropes. B) Ropes and PVC plates before deployment. C) PVC plate placed at 2m depth in June 2015 observed in May 2016. D) Location of the study area, Fangar Bay.

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

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


A
B
C
D
Fangar Bay
Ebro Delta
N
10 km
Mediterranean Sea
Atlantic Ocean


Trididemnum cereum
Clavelina lepadiformis
Clavelina oblonga
Didemnum vexillum
Styela plicata
Ecteinascidia turbinata
Placement month
19th May
16th June
14th July
11th August
9th September
Depth
0.2m
1m
2m
% cover
J J A S O N D J F M A M J J A S O N D
2015
2016


Temperature ; Salinity; Chlorophyll a
Oxygen (%)
0
5
10
15
20
25
30
35
40
0
20
40
60
80
100
120
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
2015
2016
Temperature (ºC)
Salinity (psu)
Oxygen (%)
Chlorophyll a (µg·L-1 * 10)