When invasion biology meets taxonomy: *Clavelina oblonga* (Ascidiacea) is an old invader in the Mediterranean Sea

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ABSTRACT

Taxonomic issues often confound the study of invasive species, which sometimes are unrecognized as introduced in newly colonized areas. *Clavelina oblonga* Herdman, 1880 is an abundant ascidian species along the southeastern coast of the United States and the Caribbean Sea. It was introduced into the eastern Atlantic and Brazil decades ago. In the Mediterranean Sea, a similar species had been described as *C. phlegraea* Salfi, 1929 and reported from southern Italy and Corsica. In the last few years a species of *Clavelina* has proliferated in the embayments of the Ebro Delta (NW Mediterranean), a zone of active bivalve culture industry where it has smothered mussel spat, leading to economic loss. We here report the morphological and genetic identity of this species, synonymizing the Atlantic *C. oblonga* and the Mediterranean *C. phlegraea* (the latter therefore is a synonym of the former). Thus, *C. oblonga* has existed in the Mediterranean for over 80 years, but was known under a different name. We also found this species in natural habitats in the Iberian Atlantic coast close to the Strait of Gibraltar, raising concerns about an ongoing expansion. In order to obtain information relevant for management, we monitored growth, reproductive cycles and settlement patterns of this ascidian on bivalve cultures in the Ebro Delta. Its biological cycles were markedly seasonal, with peak abundance and reproduction during the warmest months, followed by regression during the cold season. The settlement period was short, mostly concentrated in a single month each year. Avoidance of mussel and oyster seeding during late summer and early autumn can readily reduce the damage caused by this species.

Key words: ascidian, *Clavelina phlegraea*, life cycle, recruitment, aquaculture pest, fouling

INTRODUCTION

In marine ecosystems, biological invasions traditionally are associated with intense shipping around the world, introducing many alien species in ballast water, hull fouling, or navigation canals (e.g., Carlton and Geller 1993; Gollasch 2006). Aquaculture activities also have become a leading vector for introduced aquatic species (Naylor et al. 2001; Minchin 2007) which can be intentional (targeted taxa for economic purposes) or unintentional (epibiota on commercial stock) (Gollasch 2006).

Correct identification of introduced species comprises the crucial initial step for any biologically meaningful study, including applicable management (Geller et al. 1997). However, the study of invasive species in the marine realm often is confounded by taxonomic issues entailing their failure to be recognized as introduced in newly colonized areas (“pseudoindigenous species”, Carlton 2009). Upon closer scrutiny, purportedly “endemic” species, particularly in highly urbanized areas, may have been described elsewhere under different names. The long list of synonymies for some cosmopolitan species, such as the ascidians *Botryllus schlosseri*, *Styela plicata*, *Ciona intestinalis,* and *Didemnum vexillum* (Kott 1985, Lambert 2009) bears testimony to how often taxonomy has failed to cope with a global-scale perspective. This happens in part due to declining taxonomic worldwide expertise and to frequent lack of diagnostic characters. In many cases, genetic techniques have facilitated correct identification of alien newcomers, including cryptic introductions (e.g., Turon et al. 2003, McGlashan et al. 2008).

Ascidians are important marine invaders around the world (Lambert 2007), particularly in harbours, marinas, aquaculture facilities and other man-made structures (Lambert and Lambert 2003; López-Legentil et al. 2015). Although many introduced ascidians remain confined to these artificial habitats and are of little ecological concern, they can have a high economic impact to submerged infrastructures and reduce aquaculture yield.

The shellfish aquaculture industry commonly is affected by introduced ascidians worldwide, with economic losses (Fitridge et al. 2012). For example, *Ciona intestinalis* has had important impacts on bivalve cultures in Atlantic and Pacific temperate regions (Ramsay et al. 2009; Madariaga et al. 2014). *Styela clava* also is a serious pest in aquaculture facilities around the world (Goldstien et al. 2011). *Didemnum vexillum* impacts mytilid cultures and natural scallop beds (Bullard et al. 2007, Fletcher et al. 2013). Nonindigenous ascidians frequently overgrow bivalves, adding weight and restricting water exchange and nutrients, thus decreasing shellfish productivity (Daigle and Herbinger 2009). Moreover, some invasive tunicates also proliferate on natural habitats, with important community effects (reviewed in Cordell et al. 2013).

In late summer 2011, a colonial ascidian completely covered mussel spat in the Ebro Delta (Spain, northwestern Mediterranean Sea), an area of important bivalve culture activity, causing the loss of almost all juveniles, with oyster cultures also affected to a lesser extent. The ascidian recently also has been found growing on natural substrate along the shores of the Atlantic Iberian Peninsula (ca. 100 Km west of the Strait of Gibraltar). Two species descriptions matched the morphological characters of this colonial ascidian; one was *Clavelina oblonga* Herdman, 1880, native to the southern Atlantic coast of North America and the Caribbean Sea, and introduced in Brazil, Azores Islands, Cape Verde, and Senegal (Rocha et al. 2012 and references therein). Another similar species is *Clavelina phlegraea* Salfi, 1929, described from Lago Fusaro (SW Italy), which is considered to be native in the Mediterranean Sea and has been reported from lagoons in Corsica (Monniot et al. 1986), and Italy: Naples (Salfi 1929), Rome (Brunetti 1987), and Taranto (Mastrototaro et al. 2008).

In the present work, we aimed to determine the taxonomic status of the pest species *Clavelina* sp. in the Iberian Peninsula and the Mediterranean records of *C. phlegraea*, using morphological and molecular methods. As knowledge of the biology of introduced species is crucial for their management, and given its harmful effects, we also sought to determine its life cycle by analyzing abundance, reproduction, and recruitment in affected bivalve cultures. Our final goal was to provide meaningful advice for minimizing losses due to ascidian overgrowth of bivalves.

MATERIAL & METHODS

Study site and sampling

This study was conducted in the southern bay of the Ebro Delta (Alfacs Bay, Iberian Peninsula, NW Mediterranean Sea, Fig. 1). Alfacs Bay was 50 km^2 in surface area (Camp and Delgado 1987) and reached 6 m in depth, with a muddy bottom. The bay housed aquaculture facilities, with ca. 90 bivalve rafts. Each raft consisted of a rectangular structure (100 x 20 m) of wooden beams arranged in a grid, supported by cement columns. The bivalve ropes hung from the beams, and each raft contained up to 5,000 ropes. The species were the mussel *Mytilus galloprovincialis* as the main culture and the oyster *Crassostrea gigas*. Additional observations were made in the northern bay of the Ebro Delta (Fangar Bay), which was 12 km^2 (Camp and Delgado 1987) in surface area with a muddy bottom to 4.2 m depth and housed 77 rafts. Again, both bivalve species were grown in Fangar Bay, but the oyster was the most commonly cultured.

In summer 2011, the ropes of mussels and oysters in Alfacs Bay appeared heavily fouled with a clavelinid ascidian (*Clavelina* sp.), whose colonies formed balls up to 15 cm (Fig. 1). Samples were obtained in 2011-12 for morphological and genetic identification. For morphological comparison we examined colonies of *C. oblonga* previously collected by XT from Bocas del Toro (Panama), and material from Faial Island (Azores) from an earlier study (Turon et al. 2003). For morphological and genetic analyses, we also used specimens of *C. phlegraea* from the Mediterranean. Formalin-preserved samples from the Urbino Lagoon (Corsica) from the Museum National d'Histoire Naturelle (Paris) were examined. Additional samples were obtained for morphology and genetics from the Mar Piccolo of Taranto (Fig. 1). Careful search of the type locality in Fusaro Lagoon (Naples, Italy) in July 2013 by one of us (XT) did not detect any specimens. Previous attempts by collaborators likewise found none. Finally, morphological and genetic analyses were performed on specimens found in autumn 2014 on natural rocky substrate at low tide in Cadiz (South Atlantic Iberian coast), ca. 100 km west of the Strait of Gibraltar (Fig. 1).

Genetic analyses

Colonies of *Clavelina* sp. from mussel crops in Alfacs Bay (Ebro Delta, Spain) (n=27), from Cadiz (Spain) (n=7), and colonies of *C. phlegraea* from Taranto (Italy) (n=9) were fixed in 96% ethanol and stored in the laboratory at -20ºC. For DNA isolation, one zooid was dissected from each colony, and tissue from the branchial sac was extracted using a QIAamp® DNA Mini Kit (QIAGEN) and resuspended in 200 µl of AE buffer. We used just the branchial sac to avoid potential contamination from gut contents.

The universal primers HCO2198 and LCO1490 (Folmer et al. 1994) were used to amplify a fragment of the mitochondrial gene cytochrome *c* oxidase I (COI). PCR amplifications were carried out in a total volume of 20 µl with 14.7 µl H_2O, 2 µl 5x buffer (GoTaq, Promega), 1 µl $MgCl_2$ (25mM), 0.5 µl dNTP's (1 mM), 0.4 µl (10 µM) of each primer, 1U *Taq* polymerase (GoTaq, Promega) and 1 µl of DNA. PCRs began with an initial denaturation at 94 ºC for 5 min, followed by 35 cycles of denaturation at 94 ºC for 1 min, annealing at 50ºC for 1 min and elongation at 72 ºC for 1 min 30 s, with a final extension at 72 ºC for 7 min. Amplified DNA was purified with Exo-SAP and both strands were sequenced by Macrogen Inc. with the EZ-seq V2.0 service. Forward and reverse sequences were edited, aligned and confirmed visually with BioEdit sequence editor using ClustalW multiple alignment. Likewise, sequences of all individuals were aligned with haplotypes of *C. oblonga* from worldwide populations from Rocha et al. (2012).

Monitoring of abundance and growth cycle

Two approaches were used to estimate the abundance of *Clavelina* sp. over the year. First, we monitored five mussel ropes in a raft in the middle of Alfacs Bay. The ropes were placed in November 2011 and were ca. 2-3 m long, located evenly along the raft structure and facing all orientations. We monitored them monthly from December 2011 to March 2013 when they were removed by the owners (sampling could not be done in November 2012 due to logistic difficulties). For the monitoring, ropes were taken out of the water for a few minutes, laid on a flat surface, and mussels and ascidians were gently stretched out to avoid overlaps. The ropes then were photographed with a digital camera together with a ruler scale and immediately returned to sea. The perimeter of each colony in each photograph was manually outlined with Photoshop CS4, and colony areas were determined by the Laboratory of Image Analysis of the Scientific and Technological Center at the University of Barcelona. The total area of the colonies on each rope (cm^2) was divided by the total rope length (m), to obtain a relative estimate of abundance in cover area/length (cm^2/m) each month.

Second, we deployed plates to study the growth cycle. In a mussel raft located in the center of the bay we placed three ropes in December 2011, each with three PVC plates (20 x 20 cm) at three depths: 20 cm, 1m, and 2m, which were separated by tens of meters. Experiments were monitored monthly until December 2013 (except for November 2012). The PVC plates (both sides) were photographed and processed as above, except that cover was calculated as percent area of the colonies relative to the total surface area of the plate. The colonies could be easily delineated in the photographs, even if made up of a single zooid, as they form whitish masses (Fig. S1). The congeneric species *C. lepadiformis* (Müller, 1776) was occasionally present on the plates, but could be clearly differentiated by its transparent tunic and white lines in the branchial region.

Biotic and abiotic parameters of the bay were measured weekly by the staff of the Institute of Agriculture and Food Research and Technology (IRTA) as part of a long term monitoring program, including temperature (ºC), salinity, and dissolved oxygen percent saturation at 0.5 m water depth using an YSI 556 Handheld Multiparameter Instrument. Water samples were taken at the same depth and were analyzed for chlorophyll *a* with a Turner Trilogy Laboratory Fluorometer.

Reproductive cycle and recruitment

In order to study the reproductive cycle of *Clavelina* sp. in Alfacs Bay, we collected five colonies monthly from June 2012 to December 2013, with the exception of November 2012 when no collections were possible. The colonies were taken randomly, with each one from different rafts and preserved *in situ* in seawater with 10 % formaldehyde. Colonies then were dissected under a binocular microscope and ten zooids were randomly selected per colony to determine their reproductive status. Like most colonial ascidians, this clavelinid is a hermaphrodite that broods its offspring. We categorized each zooid as follows: (1) immature, (2) presence of testes, (3) presence of ovary, and (4) presence of brooded larvae. Since stages 2, 3, and 4 are not mutually exclusive, we assigned each zooid to the most advanced stage observed. A maturity index (MI) per month followed López-Legentil et al. (2005), by averaging the category numbers of ten zooids per colony and calculating the mean of five colonies.

To assess the recruitment pattern of *Clavelina* sp., we supplemented the same mussel raft where the ropes with permanent plates were located (see above) with three additonal ropes having PVC plates (20 x 20 cm) at three depths (20 cm, 1 m, and 2 m), as in the previous experiment. We replaced these plates monthly (again except November 2012), took close-up photographs, and counted the number of colonies established on them to estimate recruitment per month and depth. The new colonies could be easily counted in the photos as they were formed by a single or a few zooids.

Statistical analyses

Cross-correlation analyses were used to assess relationships of the abundance cycle and the maturity index with environmental parameters: temperature, chlorophyll *a*, salinity, and O_2 levels in the water column. Cross-correlation analysis compared two time series using the Pearson correlation coefficient, with increasing lag of one series with respect to the other (Quinn and Keough 2002). Correlations at negative lags related values in the first series to previous ones in the second. Correlations at positive lags analyzed relationships of values in the first series with future ones in the second. For the maturity index (MI), months when colonies were absent (regressed) were assigned stage 1 (immature) for cross-correlation analysis. The missing point (November 2012) was replaced by the mean of the previous and following months.

To assess coverage on the permanent (i.e., non-independent over time) PVC plates with depth, a two way repeated measures ANOVA compared months having the highest values per year (September 2012 and August 2013, see Results), using year as the within subject (plates) factor, and depth as the between-subject factor. The Kolmogorov-Smirnov test evaluated data normality and Mauchly's test the sphericity assumption (Quinn and Keough 2002). A two way ANOVA (with year and depth as factors) assessed differences in recruitment intensity on the monthly (i.e., independent over time) PVC plates, comparing months with the highest recruitment intensity per year (October 2012 and September 2013, see Results). We rank-transformed recruitment data prior ANOVA to comply with assumptions of normality (Kolmogorow-Smirnov test) and homoscedasticity (Levene test). Student-Newman-Keuls pairwise multiple comparison tests (Quinn and Keough 2002) were performed where necessary for significant factors. Statistical analyses were done using SigmaStat v 3.1 and Systat v 12.02.

RESULTS

Morphological observation

Colonies of *Clavelina* sp. from the Ebro Delta formed globular masses reaching 15 cm in diameter and 10 cm in height (Fig. 1). The masses were made of thick, anastomosed digitations of tunic coalescing towards the base, with each digitation having one to eight zooids. The tunic was soft and more consistent basally, mostly transparent with scattered whitish flecks. On the thick basal tunic there were numerous fine stolons ending in budding chambers with white pigment.

Morphological characters of the zooids and larvae are presented in Fig. 2. The zooids measured to 25 mm with some white pigment in the branchial sac and stomach, and ca. 20 simple tentacles of various orders. The neural gland aperture was shaped as a vertical oval, and there were ca. 20 rows of stigmata in the branchial sac (with 50-60 stigmata per half row in well-developed zooids). The digestive system comprised a descending esophagus and a subterminal squared stomach with marked ridges, followed by a mid-intestine and an ascending rectum. The gonads lay to the left of the intestinal loop (with the stomach located dorsally) and contained numerous ovoid and small male follicles, with a mass of oocytes in the middle of the testes. Up to 100 larvae were incubated on the right side of the posterior part of the peribranchial cavity. The distal part of the oviduct formed a dilated pouch filled with embryos that protruded postero-basally from the thorax. The fully formed larvae measured 0.8 mm. They had a well-developed ocellus and an otolith in the sensory vesicle, and bore an anterior process with three simple adhesive papillae arranged in a triangle connected by a ventral peduncle to the trunk.

Morphological characters of the colonies and zooids examined from other locations (*Clavelina* sp. from Cadiz, *C. oblonga* from Panama and Azores, *C. phlegraea* from Taranto and Corsica) were similar to each other and to those observed in *Clavelina* sp. from the Ebro Delta.

Genetic analysis

Sequence length after alignment and trimming was 658 bp. All samples from the Ebro Delta, Cadiz, and Taranto shared an identical COI sequence, which corresponded to haplotype 3 of *C. oblonga* by Rocha et al. (2012).

Growth cycle

Coverage of *Clavelina* sp. in Alfacs Bay (Fig. 3) fluctuated seasonally. Colonies did not appear on the five mussel ropes deployed in November 2011 until July 2012 (24.49±2.56 cm^2/m, mean±SE), reached maximum coverage in September 2012 (706.26±328.07 cm^2/m), and then regressed to almost disappear by the end of January 2013 (0.41±0.25 cm^2/m). By the next (and final) observation in March 2013, they had completely disappeared. Mussel ropes were removed by fishermen and thus monitoring ended in March 2013.

Results from monitoring the three ropes with permanent PVC plates at three depths, shown in Fig. 3, are consistent with findings for the mussel ropes. In both, the month with highest cover was September 2012, when the ascidian almost completely occupied the plates at 1 m (93.36±6.64%, mean±SE) and 2 m (98.51±1.49%), with significantly less coverage at 20 cm (45.03±1.54%), likely due to the prevalence of the solitary ascidian *Styela plicata*. In October 2012 ascidian cover was reduced to less than 10% at the three depths and by January 2013 was just 1%. The colonies completely regressed afterwards, and were absent until April 2013 when the ascidian reappeared, albeit with less than 1% cover. Active growth did not begin until July/August. In 2013 coverage was less than the previous year, and was maximal in August, reaching 47.58±10.20% cover at 2 m. By then, shallower plates were completely covered by *Mytilus galloprovincialis* with few ascidian colonies (13.34±5.01% cover). The ascidian then regressed again, barely reaching 4% cover at any depth in December 2013. Repeated-measures ANOVA (Table 1) showed a significant year effect, with greatest cover in 2012. Depth also was significant, showing no significant interaction depth*year. Student-Newman-Keuls tests indicated that the shallower plates (at 20 cm) had significantly less cover than the deeper ones (1 and 2 m).

The time course of environmental variables (temperature, salinity, levels of O_2 and levels of chlorophyll *a*) over the study period is in Figs. 3 and S2. Overall, although temperature showed a clear seasonal pattern with some interannual differences (e.g., lower 2012 winter temperatures and cooler 2013 spring), other variables had no clear-cut patterns. Weekly water temperatures (at 0.5 m) ranged from 5.71°C in February to 28.95°C in August in 2012 and from 8.56°C in February to 28.74°C in July during 2013.

Relationship of these variables to ascidian abundance was evaluated with cross-correlation analysis of mean coverage of the PVC plates (Figs. 4 and S3), which showed positive significant correlation with temperature for the current and two previous months (lags of 0, -1, -2, Fig. 4). Correlations were significantly negative at intervals of 4-7 months, reflecting seasonal nature of both variables. Chlorophyll *a* was significantly correlated with coverage of the current month (Fig S3), which is attributable to the September 2012 peak (Fig. S2) coinciding with the *Clavelina* sp. bloom. Coverage was significantly negatively correlated with salinity of upcoming months (lag +4, Fig. S3). Likewise, a negative significant correlation occurred between coverage and oxygen levels of the previous month (lag -1, Fig. S3). We could not assign any clear biological meaning to correlations with oxygen levels or salinity, which may be random data outcomes.

Reproductive cycle and recruitment

Reproduction in *Clavelina* sp. was strongly seasonal. Brooding larvae were observed during summertime and early autumn, peaking in August and September (Fig. 5). From December 2012 to April 2013, colonies remained immature or absent (February and March 2013).

The Maturity Index (MI) increased during summer 2012, reaching its highest in September (Fig. 5) and diminishing afterwards, with all colonies immature by December. In February and March 2013 there were no colonies. In April 2013 we found just a few small immature colonies. MI increased again in spring 2013, with brooding colonies apparent in July 2013. In 2013 MI was highest in August, which was lower than the previous year (Fig. 5). Only a few zooids still had larvae in November and again all colonies were immature in December 2013.

Patterns of MI and temperature appear to match, with cross-correlation analysis showing a significant positive correlation at time lag 0, as well as at the two previous months and the following one (lags -1, -2, +1; Fig. 4). Moreover, MI was significantly positively correlated with the present and previous months' salinity (Fig. S4), while levels of O_2 and chlorophyll *a* were uncorrelated with MI (Fig. S4). On the other hand, MI also was significantly correlated with the coverage of the present, previous, and following months (lags -1, 0, +1, Fig. 4).

Some recruitment occurred on the plates during summer 2012 (reaching mean values of ca. 14 recruits per plate in July), peaking markedly in October to more than 100 recruits per plate at 20 cm (Fig. 6). In December 2012 only three recruits occurred in total. No recruitment was observed afterwards, until July 2013, when a single recruit was found (at 2 m). In 2013, recruitment was more intense than the previous year and peaked in September, with over 300 recruits per plate at 2 m (Fig. 6). Recruitment decreased afterwards and in November 2013 there were only ca. 10 recruits per plate at any depth. In December 2013 there was no further recruitment. Interestingly, recruitment was greater in the shallowest plates in 2012, a pattern opposite that of 2013, when it was highest at 2 m (Fig. 6). ANOVA results (Table 1) showed a significant interaction term, due to the different pattern of recruitment with depth between the two years. Comparisons (Student-Newman-Keuls tests) at fixed levels of the factor depth revealed higher recruitment in 2013 at all depths, whereas there was significantly less recruitment at 2 m in 2012, and no depth-related differences in 2013.

DISCUSSION

Morphological analyses showed that *Clavelina* sp. from the Mediterranean (Ebro Delta) and the Atlantic (Cadiz) Iberian coasts matched previous descriptions of *C. oblonga* (e.g., Van Name 1945) and *C. phlegraea* (e.g., Brunetti 1987), as well as examined material from Panama and Azores (*C. oblonga*), and two of the four locations where *C. phlegraea* has been reported (Mar Piccolo of Taranto and Urbino Lagoon). Further, our genetic analyses indicated that *Clavelina* sp. from the Ebro Delta and Cadiz, and *C. phlegraea* from Taranto have the same *COI* haplotype, which also characterizes introduced populations of *C. oblonga* (Rocha et al. 2012). We conclude, therefore, that *C. oblonga* and *C. phlegraea* are the same species, with the former name having precedence. It is unfortunate that the type specimen of *C. phlegraea* is unavailable and that no material from the type locality (Fusaro Lagoon, SW Italy) could be found, despite repeated attempts and a thorough survey. This is hardly surprising, though, given the history of Fusaro Lagoon in recent decades, with increased pollutants along with intense dredging in the 1980s (De Pippo et al. 2004). It seems that this species does not exist anymore in the phlegraean fields for which it was named (Salfi 1929).

Clavelina oblonga, described from Bermuda, is considered indigenous in the tropical western Atlantic Ocean (South Carolina to Panama), from where it spread south (southern Brazil) and east (Azores, Cape Verde, Senegal) (Rocha et al. 2012). These introductions were detected in Cape Verde by Hartmeyer (1912), along the African Coast by Pérès (1951), and in Azores by Monniot (1974). In Brazil *C. oblonga* was known since 1925 (Rocha et al. 2012). It was unreported in the Mediterranean prior to our findings, but was known under a different name from at least 1929, as a pseudoindigenous species (Carlton 2009). Its exclusive occurrence exclusively in lagoons having mariculture activities should have raised suspicions about its non-native status.

The finding of this species in the southwest Iberian Peninsula (Cadiz) represents its first report from European Atlantic shores. This raises concerns about its invasive potential, as it occurred in the shallow subtidal of an open-shore rocky locality, without nearby aquaculture facilities. It therefore appears that *C. oblonga* has the potential to spread to natural habitats along the open coast. Although the effect of *C. oblonga* on natural biota remains untested, introduced ascidians can have important impacts on natural communities (e.g., *Pyura praeputialis*, Castilla et al. 2004, *Didemnum vexillum*, Bullard et al. 2007).

Genetic composition of *Clavelina oblonga* populations is consistent with their introduction history from the tropical West Atlantic. Despite overall low diversity, four *COI* haplotypes occurred in Caribbean waters, with just one in the putatively introduced regions of southern Brazil and the Azores (Rocha et al. 2012). This haplotype was the only one found in this study.

According to local farmers, *C. oblonga* was present in the Ebro Delta some 3-4 years before its 2011 bloom, and in subsequent years we observed high summer abundances. It likely was introduced from Italy, a common source of mussel spat in Ebro Delta cultures. Again according to local farmers, it poses a threat to mussel cultures (*M. galloprovincialis*), which can be completely smothered, and slows growth of oysters (*C. gigas*). In 2012, farmers started noticing proliferation of *C. oblonga* in Fangar Bay, the northern bay of the Ebro Delta (also with important aquaculture facilities).

Clavelina oblonga in the Ebro Delta showed a markedly seasonal life cycle, with abundance and reproduction peaks coincident with the warmest months. Noticeable differences in cover values were found between sampling years. Since ropes and plates for monitoring growth were laid in November and December 2011, the fouling community was well developed by the time colonies appeared in July/August, which had to be from new recruitment. On the other hand, colonies developing on the plates the following year could comprise those surviving the winter in the form of dormant buds and/or new recruits. Temperature was significantly positively correlated with growth (cover) and reproduction (MI). Brooding of larvae occurred mostly during mid-summer, followed by their mass release with recruitment peaking on the plates in September and October, depending on the year. Water temperature has been shown to critically affect the growth cycle of colonial ascidians (e.g., De Caralt et al. 2002; López-Legentil et al. 2005). Nevertheless, peak chlorophyll *a* in the Bay also was coincident with the warmest months in 2012 (and to a lesser extent in 2013), so increased food also likely increases growth and reproduction. Further evidence for temperature effects came from an abnormally cold 2013 spring, which delayed the early summer temperature rise (up to four degrees less in June 2013 compared to June 2012); this delayed zooid maturity and lowered MI (Fig. 5).

In the Mediterranean, *C. oblonga* occurs at its northern extreme. In Alfacs Bay water temperature ranges from less than 10ºC in winter to about 28ºC in summer. Winter conditions likely are too harsh for this tropical species, which regresses in this season. Regression during unfavorable periods is common in clavelinids (De Caralt et al. 2002), and resting buds ensure population recovery when favorable conditions return. Among the biological studies of this species, Rocha (1991) and Mastrototaro et al. (2008) also found increased abundances in the warmest months in Brazil and Mar Piccolo of Taranto (referring to the species as *C. phlegraea*).

The important invasive species *D. vexillum* is another recent introduction in the Ebro Delta, detected during the monitoring of *C. oblonga*. Comparing the abundance patterns of both species reveals the varying role of temperature. *D. vexillum* is a successful invader in cold-temperate regions worldwide (Lambert 2009, Stefaniak et al. 2012) and is limited by high summer temperatures in the study area. Its life-cycle accordingly is reversed with respect to *C. oblonga*. *D. vexillum* regresses in summer and grows actively in winter-spring (Ordóñez et al. 2015). Thus, different invader species may monopolize substrate at different seasons according to their distributional affinities (either tropical or temperate), creating a mixture of lifestrategies. This can explain why *D. vexillum* is more abundant in Fangar Bay (north side of the river), which has slightly lower summer temperatures.

Clavelina oblonga was more abundant at one and two metres in the permanent plates than on the shallower ones. Competition with other species that also grew on the plates near the surface (such as *Styela plicata*) could explain this pattern. Moreover, coverage on permanent PVC plates was significantly lower in the second monitoring year, when there was an almost two-year-old community on the plates (including other ascidians, mussels, bryozoans, polychaetes, algae, and sponges), and temperature was somewhat different (a cooler spring than the previous year). Although biotic and abiotic effects on *C. oblonga* remain little studied, previous work showed temperature and biotic interaction effects on the early life stages of other invasive ascidians (*S. plicata* and *Microcosmus squamiger*; Pineda et al. 2012; Ordóñez et al. 2013). Thus, it is likely that both temperature and competitors regulate the distribution and abundance of *C. oblonga* on our ropes. Clearly, *C. oblonga* can become a dominant competitor for space, but apparently needs an initial growth period under favorable conditions (Rocha 1991).

In this study we have shown how taxonomic problems can confound studies of invasion biology, and unmasked an old introduction to the Mediterranean that is causing losses in bivalve cultures. We also provided information about the life cycle of *C. oblonga*, and showed that, as a species with tropical affinities, its populations bloom in summertime. Learning about the biology of introduced species is crucial in order to achieve successful management. Timing mariculture activities to the ascidian’s life cycle can greatly reduce its negative impact. The 2011 bloom was caused by the fishermen’s attempt to obtain an extra cohort of marketable mussels, involving mussel re-seeding in July, which became completely covered whit mass *C. oblonga* recruitment in September. Our results suggest that farming in July and waiting until mid-autumn for placing new spat is the best option to minimize damage due to summer proliferation of *C. oblonga*. Indeed, this was the traditional schedule of local farmers and we strongly advocate its maintenance. Overall, our work illustrates the importance and usefulness of correctly identifying introduced species and employing basic life history knowledge for correct management to mitigate their impact.

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Table 1. (A) Two way repeated measures ANOVA to assess differences in coverage on permanent plates with depth (for months with the highest values, September 2012 and August 2013). (B) Two way ANOVA to assess differences in recruitment intensity on monthly plates with depth (for months with the highest recruitment intensity, October 2012 and September 2013).

(A) Two Way Repeated Measures ANOVA (Coverage on permanent plates)

	DF	**SS**	**MS**	**F**	**P**
Between subjects					
Depth	2	0.690	0.345	27.140	**<0.001**
Error	6	0.076	0.013		
Within subjects					
Year	1	0.892	0.892	102.493	**<0.001**
Year x Depth	2	0.037	0.019	2.131	0.200
Error	6	0.052	0.009		

(B) Two Way ANOVA (Recruitment on monthly plates)

	DF	**SS**	**MS**	**F**	**P**
Year	1	528.321	528.321	53.562	**<0.001**
Depth	2	83.574	41.787	4.236	**0.043**
Year x Depth	2	232.170	116.085	11.769	**0.002**
Error	11	108.500	9.864		

Figure 1. (A) Sampling and monitoring site of *Clavelina* sp. in Alfacs Bay (Ebro Delta, Spain, western Mediterranean Sea, 40º37’01”N, 0º37’26”E), Cadiz (Spain, Atlantic Iberian coast, 36º31’59”N, 6º18’41”W) and Taranto (Italy, eastern Mediterranean Sea, 40º28’36”N,17º15’5”E). (B) *Clavelina* sp. overgrowing oyster crops and (C) mussel crops in Alfacs Bay (Ebro Delta, Spain).

Figure 2. Morphological features of *Clavelina* sp. in the Ebro Delta. A) distal part of a zooid, B) gonads in the basal abdomen, C) larvae, D) dissected branchial sac (ap: adhesive papillae, bp: brooding pouch, bs: branchial sac, ng: aperture of neural gland, o: ovary, sr: stigmata rows, sv: sensory vesicle, te: testes, tu: tunic). Scale bars: A, B, D, 1mm; C, 0.5 mm.

Figure 3. Growth cycle of *Clavelina* sp. in Alfacs Bay. Values are mean percent cover on PVC plates (n=3) at each depth (grey lines), and mean monthly coverage (in cm^2 of ascidian per m of rope) on the five mussel ropes (black line). Bars are standard errors. Temperature time-course (monthly means of weekly observations) is presented.

Figure 4. Cross-correlation analyses relating the mean monthly coverage (mean percentage cover of the permanent PVC plates) and Maturity Index (MI) of *Clavelina* sp. with temperature. Cross-correlation between MI and coverage is provided. Data series were lagged with respect to one another and the Pearson correlation coefficient computed for each time lag (months). Curved lines represent the threshold for significant (p=0.05) correlation values.

Figure 5. Reproductive status and Maturity Index (MI) of *Clavelina* sp. during the monitoring period. Columns indicate the percent of zooids in each stage. Note that stages are not exclusive and the most advanced one is assigned to each zooid (see text). No data were available for November 2012. In February and March 2013 there were no colonies (grey columns). Bars in MI are standard errors.

Figure 6. Recruitment of *Clavelina* sp. on the PVC plates replaced monthly at the three depths. Bars are standard errors. The time-course of temperature (monthly means of weekly observations) is presented.

Figure 1

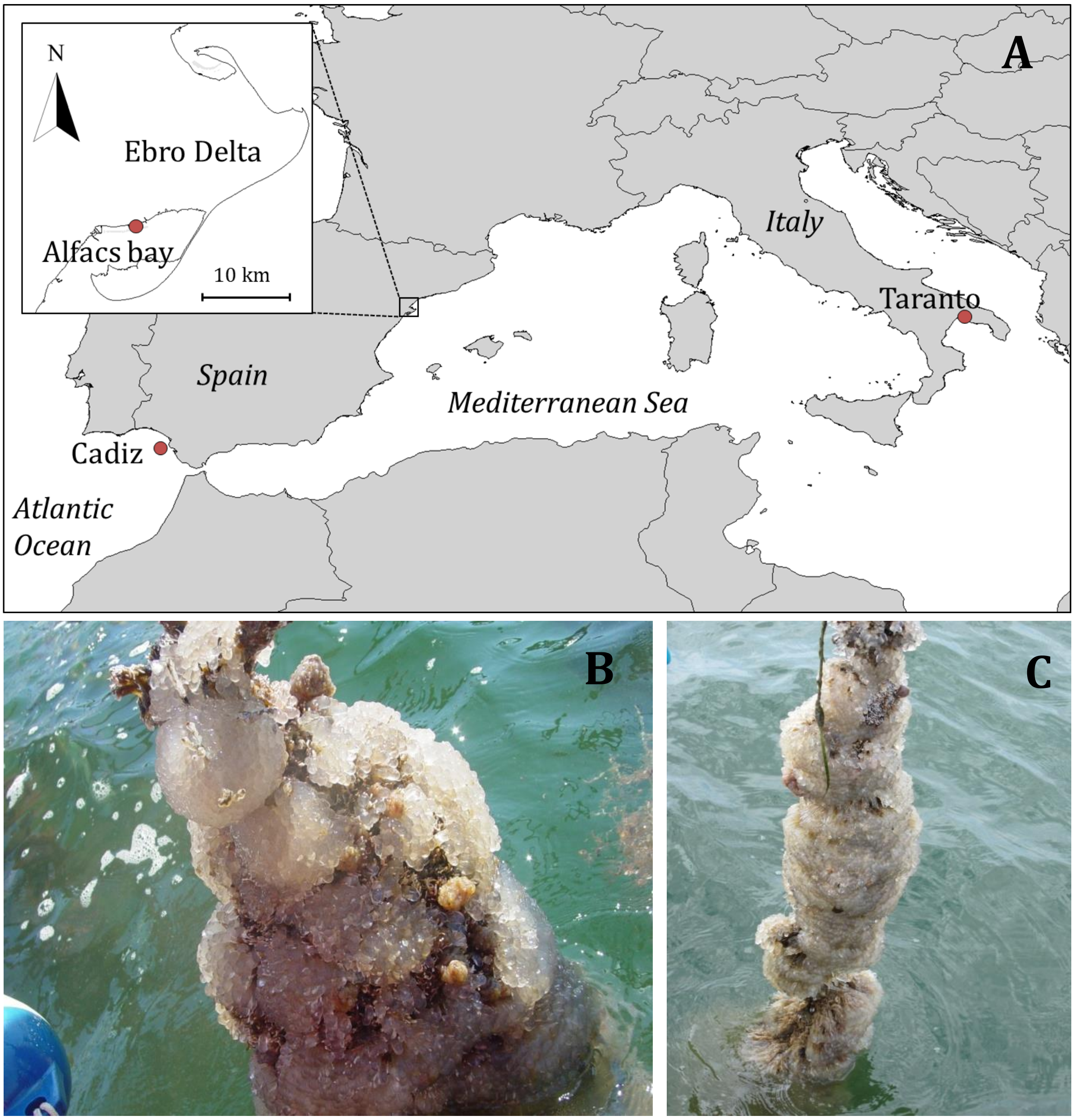

A
N
Ebro Delta
Alfacs bay
10 km
Italy
Taranto
Spain
Mediterranean Sea
Cadiz
Atlantic
Ocean
B
C

Figure 2

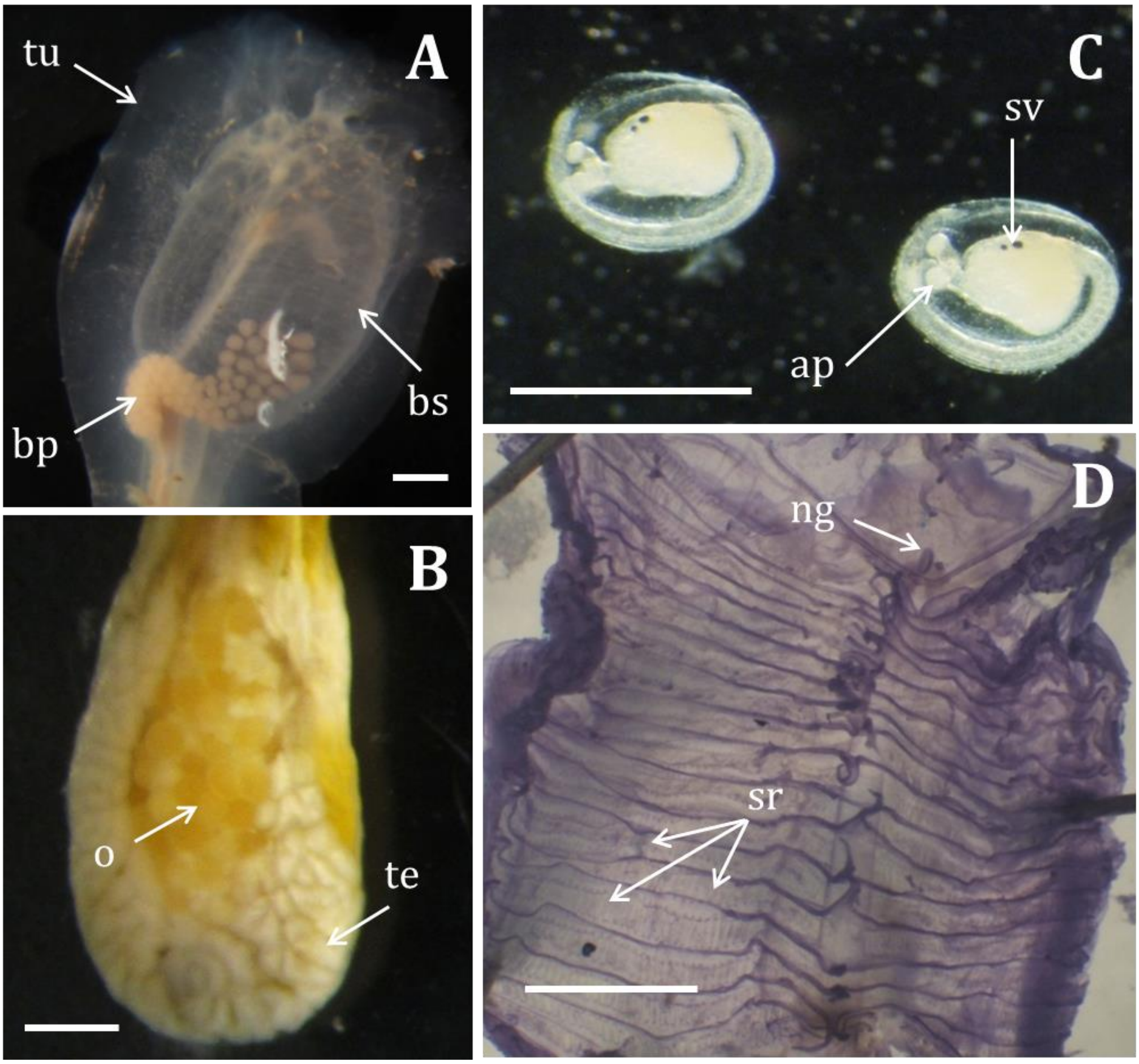


Figure 3

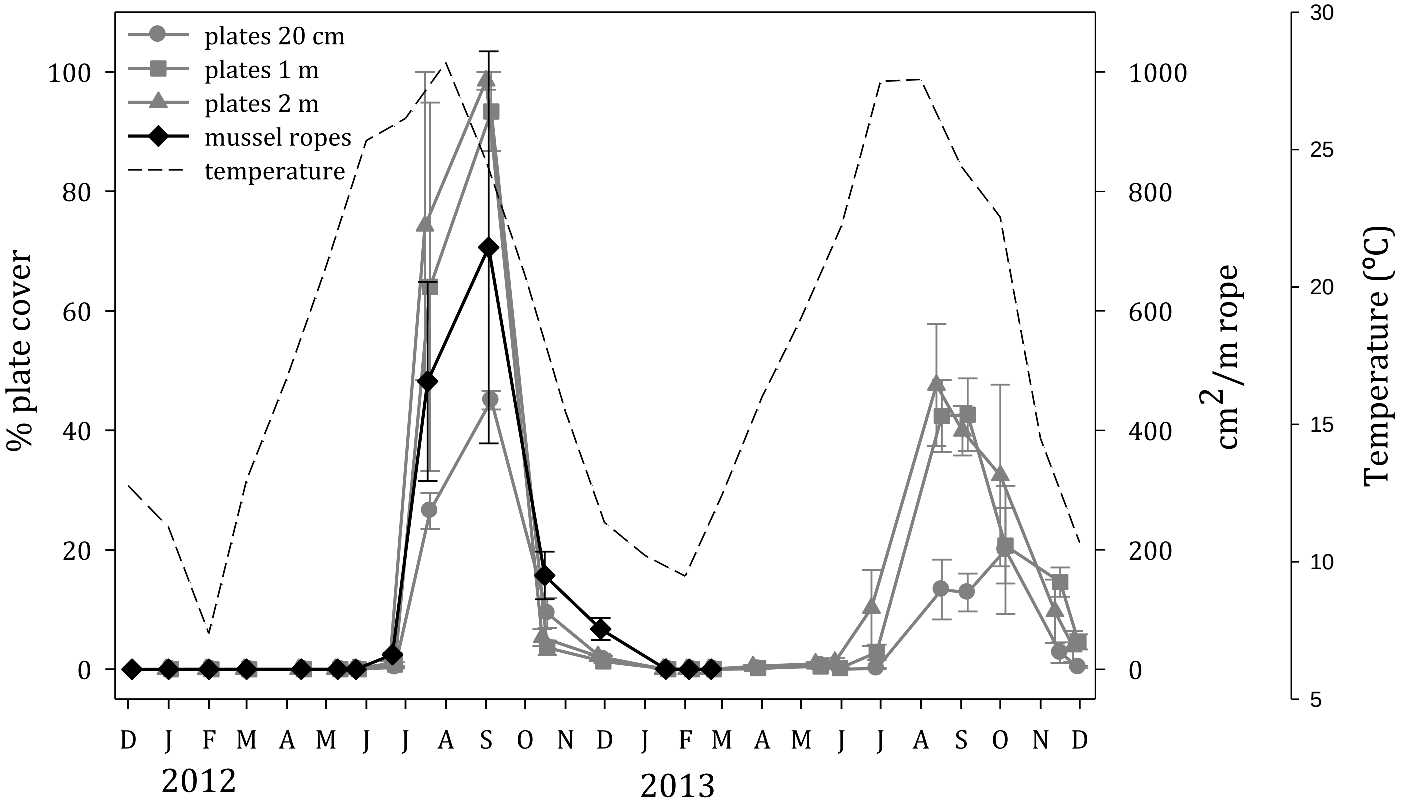

plates 20 cm
plates 1 m
plates 2 m
mussel ropes
temperature
% plate cover
0
20
40
60
80
100
cm^2/m rope
0
200
400
600
800
1000
Temperature (ºC)
5
10
15
20
25
30
D J F M A M J J A S O N D J F M A M J J A S O N D
2012
2013

Figure 4

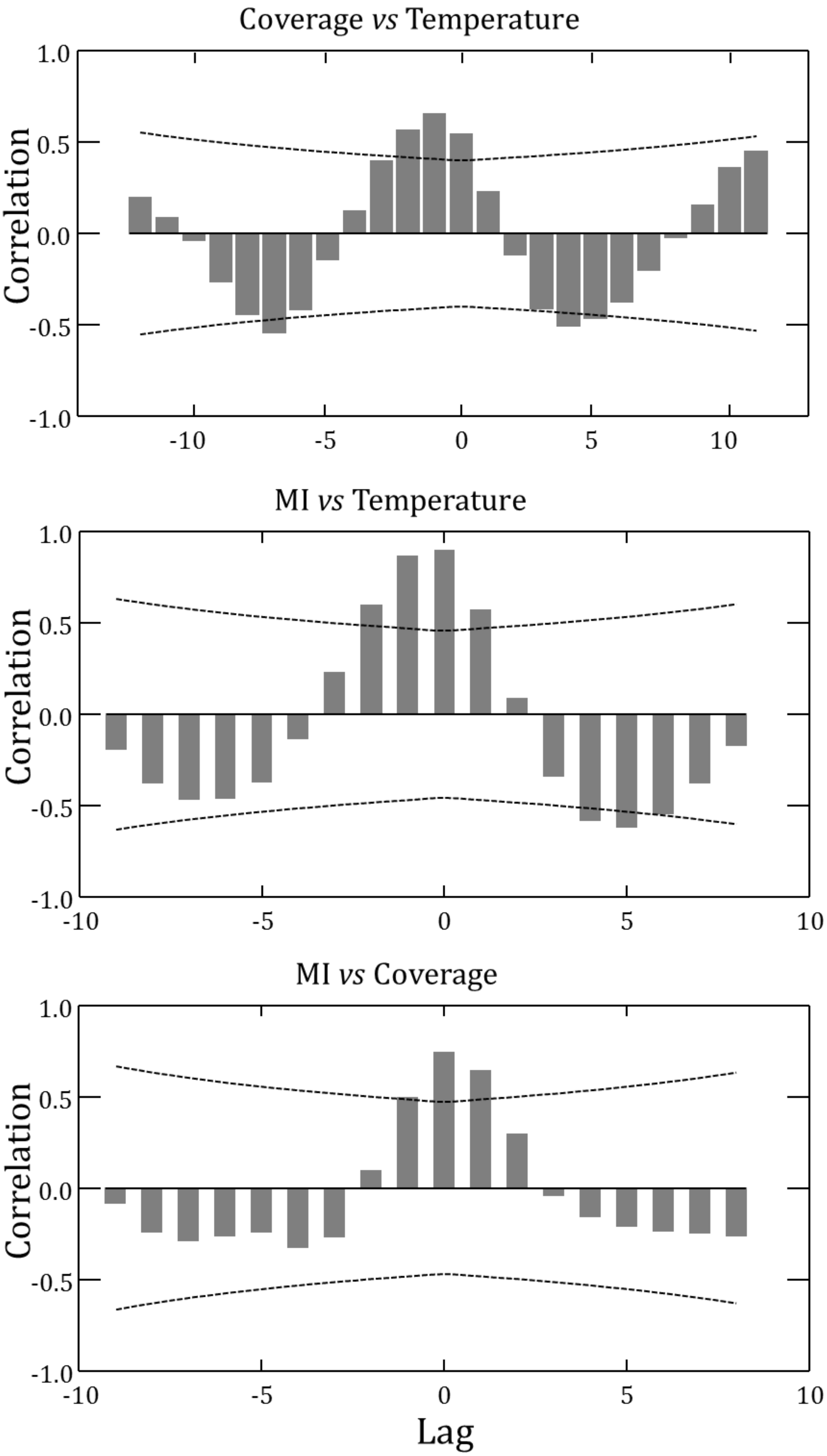

Coverage *vs* Temperature
1.0
0.5
0.0
-0.5
-1.0
Correlation
-10
-5
0
5
10
MI *vs* Temperature
1.0
0.5
0.0
-0.5
-1.0
Correlation
-10
-5
0
5
10
MI *vs* Coverage
1.0
0.5
0.0
-0.5
-1.0
Correlation
-10
-5
0
5
10
Lag

Figure 5

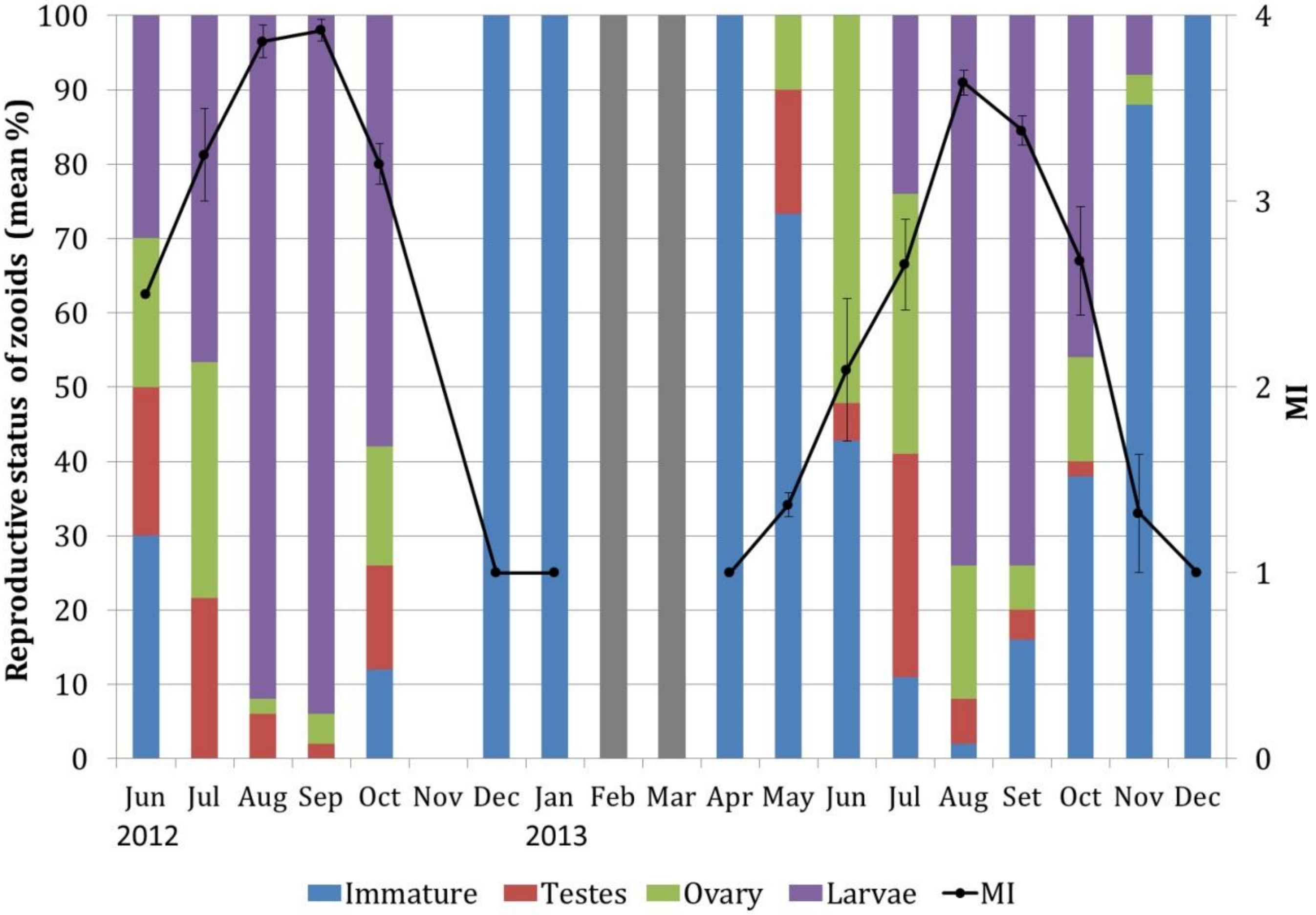
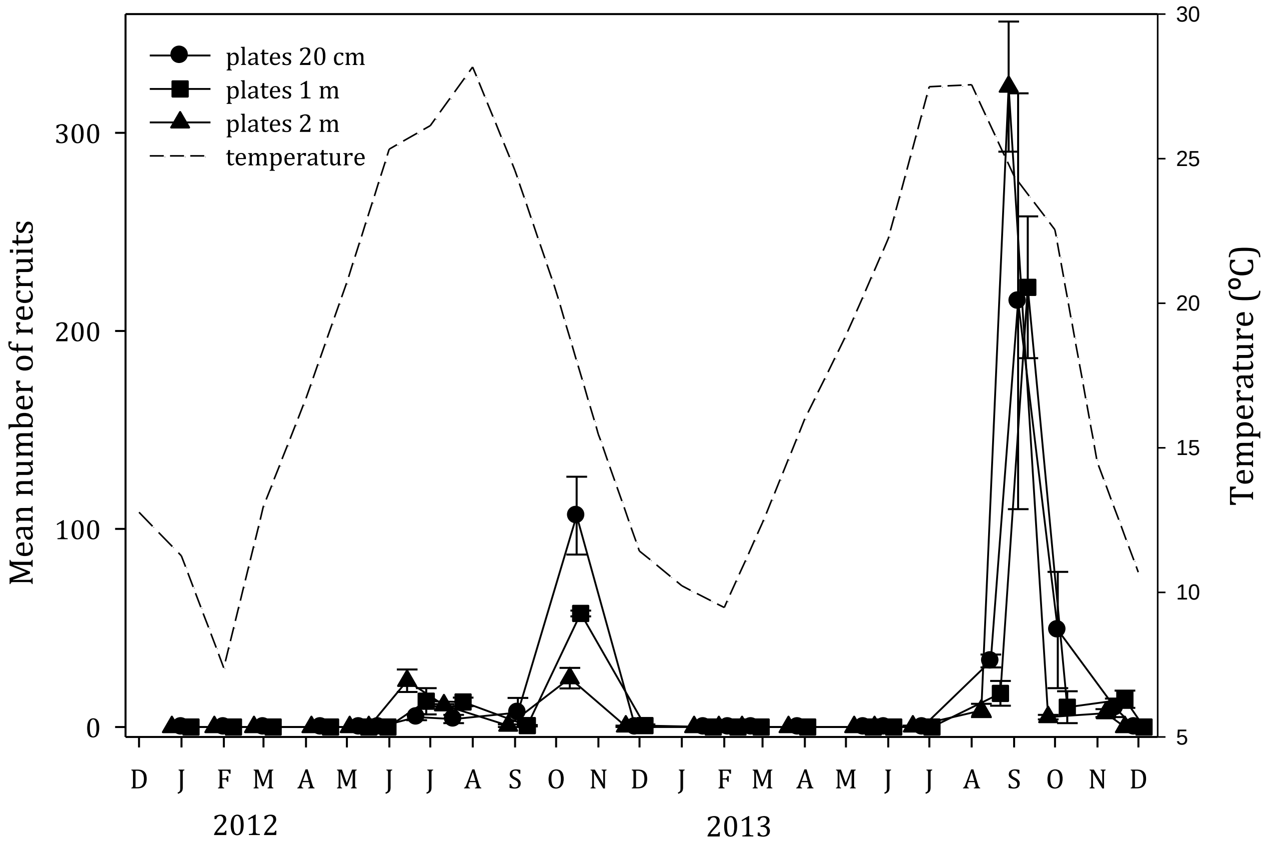

Reproductive status of zooids (mean %)
100
90
80
70
60
50
40
30
20
10
0
MI
4
3
2
1
0
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Set
Oct
Nov
Dec
2012
2013
Immature
Testes
Ovary
Larvae
MI

Figure 6


plates 20 cm
plates 1 m
plates 2 m
temperature
Mean number of recruits
Temperature (ºC)
0
100
200
300
5
10
15
20
25
30
D
J
F
M
A
M
J
J
A
S
O
N
D
J
F
M
A
M
J
J
A
S
O
N
D
2012
2013

ELECTRONIC SUPPLEMENTARY MATERIAL

Biological Invasions

When invasion biology meets taxonomy: presence of *Clavelina oblonga* Herdman, 1880 in the Mediterranean Sea

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SUPPLEMENTARY FIGURES

Figure S1. Image of a permanent plate in August 2012 (left, note recruits of *Styela plicata* indicated by arrows), and the same plate with the outline of the colonies of *Clavelina* sp. marked for area measurements (right).

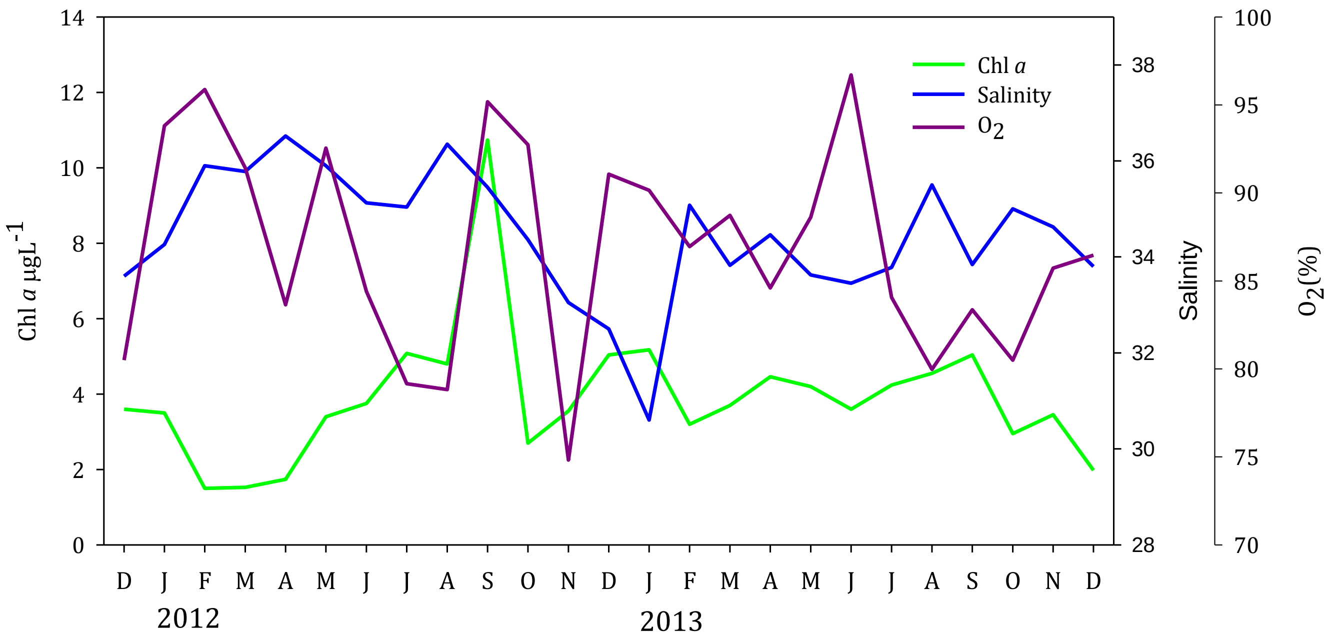
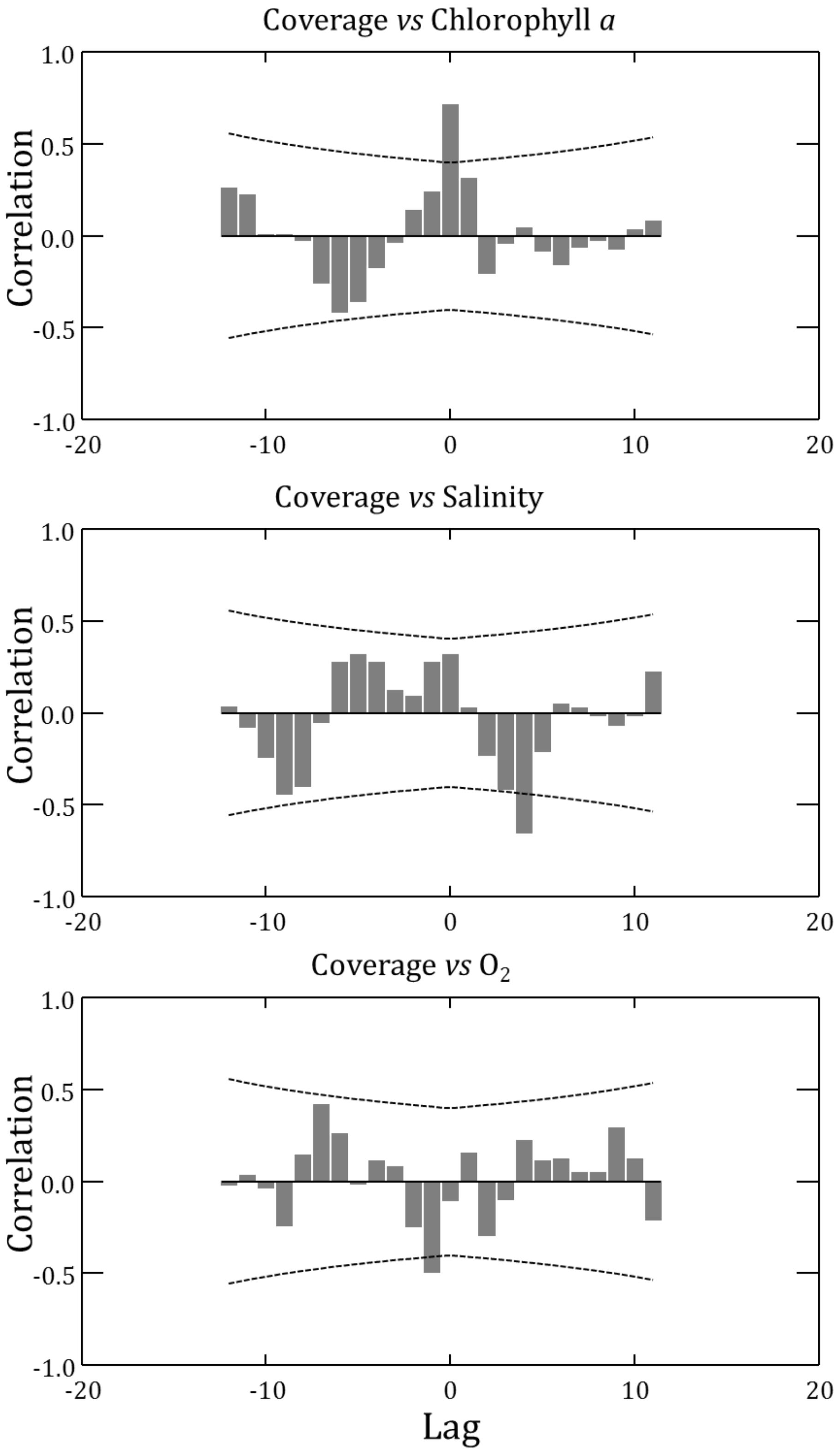


Figure S2. Environmental parameters (levels of chlorophyll *a*, salinity, and oxygen) in Alfacs bay (Ebro Delta, Spain) during the monitoring period. Data are monthly averages of weekly measures.



Fgure S3. Cross-correlation analyses relating the mean monthly coverage (mean percent cover in the permanent PVC plates) of *Clavelina* sp. with chlorophyll *a* (μgL^{-1}), salinity, and levels of O_2 (%) in the water column. Data series were lagged with respect to one another and the Pearson correlation coefficient computed for each time lag (months). The curved lines represent the threshold for significant (p=0.05) correlation values.

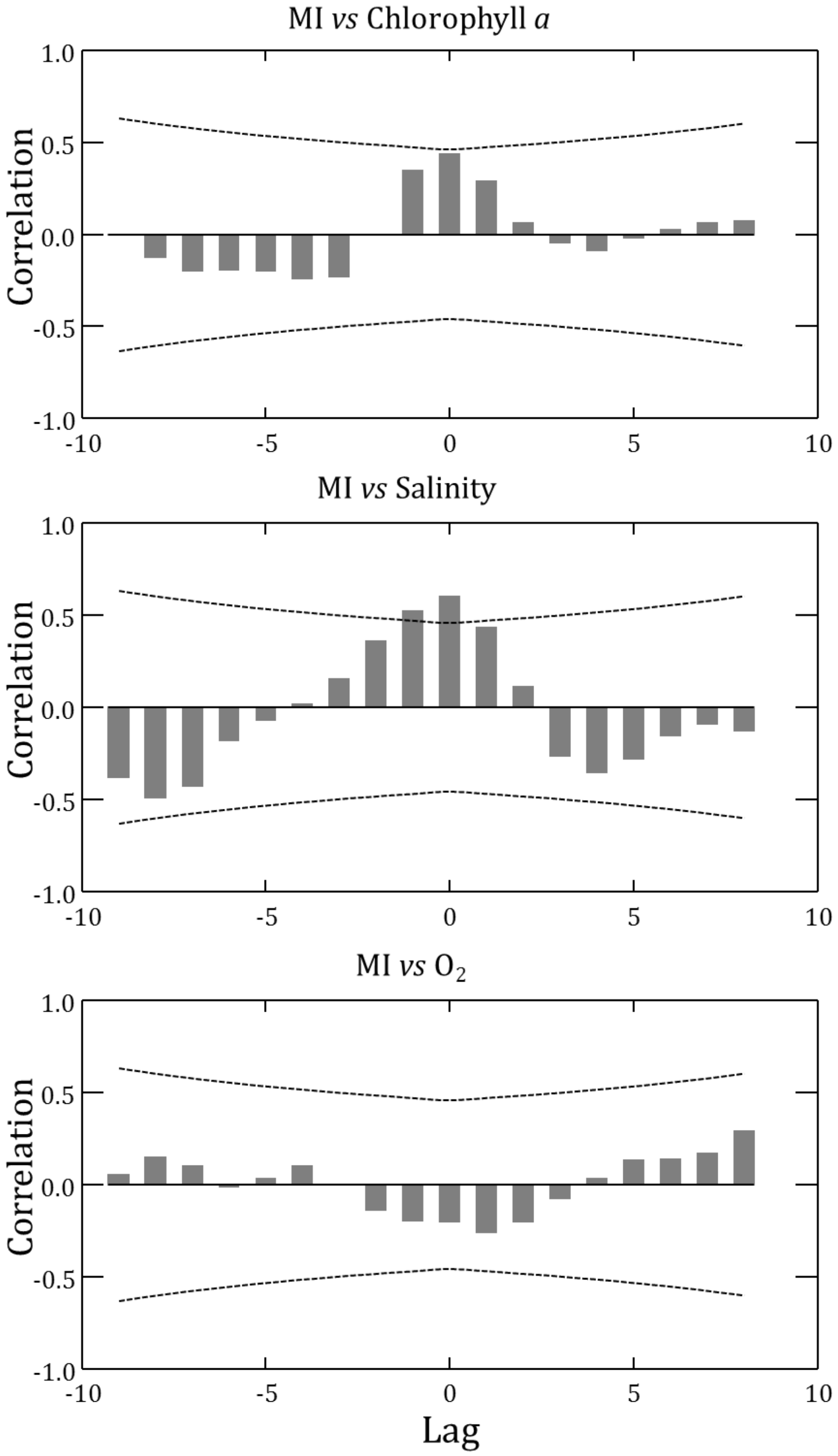


Figure S4. Cross-correlation analyses relating the Maturity Index (MI) of *Clavelina* sp. with chlorophyll *a* (μgL^{-1}), salinity, and levels of O_2 (%) in the water column. Data series were lagged with respect to one another and the Pearson correlation coefficient computed for each time lag (months). The curved lines represent the threshold for significant (p=0.05) correlation values.