

Article

Effect of Predation on Fouling Communities in an Italian Hotspot of Non-Indigenous Species

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Abstract: The role of native predators in controlling or facilitating non-indigenous species (NIS) growing in a fouling assemblage was investigated with a 70-day caging experiment in a marina inside the Gulf of La Spezia (Italy). Submerged PVC panels were divided into three experimental treatment levels: “control panels”, “open-caged panels” and “caged panels”, to assess the predation effect on the fouling assemblages growing on the panels. Predation effect was observed for two investigated variables. The Shannon Index was significantly higher in caged panels in comparison with controls, while an opposite trend was observed for the coverage area of native species. When testing single NIS abundance, both facilitation and limitation of individual NIS settlement has been observed. Mean coverage of the sabellid *Branchiomma luctuosum* was significantly higher in caged than in uncaged or open-caged panels, while the ascidian *Styela plicata* was prevalent in open-caged panels. In general, a predation effect was observed when considering the whole fouling assemblage, including different trends in NIS coverage among treatment levels. The results of this first attempt performed in the Mediterranean Sea encourage further experiments to investigate the biotic resistance provided by native predators against NIS.

Keywords: macrozoobenthos; experimental ecology; bioinvasions; Western Mediterranean Sea; ports; predator exclusion



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1. Introduction

Consensus has grown about the importance of predation on community structure and species diversity [1–3]. The spread of non-indigenous species (NIS), a process which has intensified in recent years, especially in the Mediterranean Sea [4,5], provides insight into the role of predation in the interactions between NIS and native species [6]. Non-indigenous species, i.e., species transported by anthropogenic means outside their native range, are generally exposed to strong environmental pressures when they arrive in a new region. Only those NIS that are able to adapt to the biotic and abiotic variables in the new environment and outcompete native organisms can survive and establish self-sustained populations [7–9]. Furthermore, the co-evolved predators and parasites of the introduced NIS may be absent in the recipient region, thus facilitating their establishment [10,11].

A manipulative field experiment in a Mediterranean environment can add insight into the importance of predation on NIS development.

In recent years, several studies have been carried out in order to assess the role of native predators in facilitating or limiting NIS colonization success, mainly in undisturbed natural sites, (e.g., rocky shore habitats in Marine Protected Areas), considering the possible means of interaction. For example, a prevalent feeding pressure of native predators on native species could favor the spread of NIS. NIS can also be facilitated by intrinsic characteristics, such as fast growth and high fecundity, or by local environmental conditions and by the structure of the recipient assemblages [12–14]. On the contrary, a limiting effect of native predators on NIS spreading has also been observed [15,16]. A potential biocontrol of non-indigenous fishes by high-level native predators has been also suggested [17,18].

Unfortunately, few studies on food webs have assessed the trophic positions of NIS in comparison with native species, but two examples from the Mediterranean Sea highlighted a possible ecological alteration in native communities after the inclusion of NIS as a new food source or as a new competitor/predator. Marić et al. [19] reported the food source contribution to the diets of native and non-indigenous fishes and macroinvertebrates, observing that the additional food source provided by the non-indigenous alga *Caulerpa cylindracea* Sonder may facilitate herbivorous NIS. Furthermore, the presence of a NIS can overlap the trophic position of different native species during its ontogeny, causing important ecological impacts (Mancinelli et al. [20] on *Callinectes sapidus* Rathbun, 1896). The presence of non-indigenous predators can induce a trophic cascade, facilitating the establishment of other NIS in the prey communities [21]. This effect can be more pronounced in systems with a simple food web, as in temperate areas, while at lower latitudes a stronger consumer pressure and top-down control, due to their trophic complexity, could be more robust against alterations by NIS [8]. In general, an increment in predation in tropical waters intensifies the effects on the sessile marine communities, strongly altering the structure of the prey community, as demonstrated in a large-scale experiment conducted on fouling communities [22,23]. This could explain the comparatively lower success of NIS colonization at low latitudes [24,25], but further experiments are surely required to confirm these and other hypotheses on the role of biological interactions in NIS success [26].

Fouling communities can dominate in anthropogenic habitats [27] and are a good model for testing ecological questions, being easy to manipulate in order to document bioinvasion processes [23]. Predation effects for marine urbanized areas have often been assessed on target species only, for which generally lower growth and survivorship was recorded in the presence of predators, as, for example, on barnacles in the Gulf of Mexico [28] or ascidians in Brazil [29]. Since the mid-1990s, several local-scale studies have also been carried out considering the effects of predators on NIS, e.g., laboratory experiments to assess the potential predators of non-indigenous colonial ascidians in British Columbia [30]; a caging experiment in Argentina which recorded a strong colonization of non-indigenous ascidians when benthic predators were excluded, even if nektonic predators were not [31]; and in Chile, a reduction of the abundance of most NIS was observed considering both micro- and macropredators [32]. The predation pressure in urbanized areas could be different from that in natural environments. A general reduced richness can be observed in both environments, but to a lesser extent in fouling communities from artificial areas; possibly depending on latitude [33], different composition of predators observed in the two habitat types [34,35] or biological traits of the species considered [36].

Several studies around the globe have underlined different effects of predation in determining the composition of fouling assemblages, or on single target species, namely a facilitation or limitation role on NIS settlement. In particular, a direct effect of native predators against NIS colonization has been observed in several studies (e.g., [37–39]), but predators can also have an indirect effect on NIS facilitation by consuming native species [32,40].

In this study, a manipulative experiment was carried out to assess the role of macro predators in limiting or facilitating NIS colonization in fouling communities growing on experimental polyvinyl chloride (PVC) plates. The experiment was conducted in a Mediterranean marina (Gulf of La Spezia, Ligurian Sea, Italy) with the use of exclusion cages, testing differences in the composition of fouling communities between treatments (caged, vs. uncaged panels) [25,32].

2. Materials and Methods

2.1. Study Area

This experiment was conducted in Santa Teresa Bay (44°4'53.998'' N–9°52'54.538'' E; Figure 1A) in late summer 2020, for a total of 70 days (from August to October). Santa Teresa Bay is a small, natural, rocky shore embayment in the Gulf of La Spezia (Ligurian Sea, Italy). One side of the bay features a marina with a small concrete dock and a floating

pontoon, hosting a few recreational boats, while on the other side there is a concrete dock used only by small fishing boats to carry mussels from the nearby aquaculture facility. Despite these anthropogenic impacts, the bay is embedded in a natural environment and is popular as a tourist spot in the Gulf of La Spezia, but it is also known to host a high incidence of NIS in fouling communities, probably due to the presence of the aquaculture facility [41].

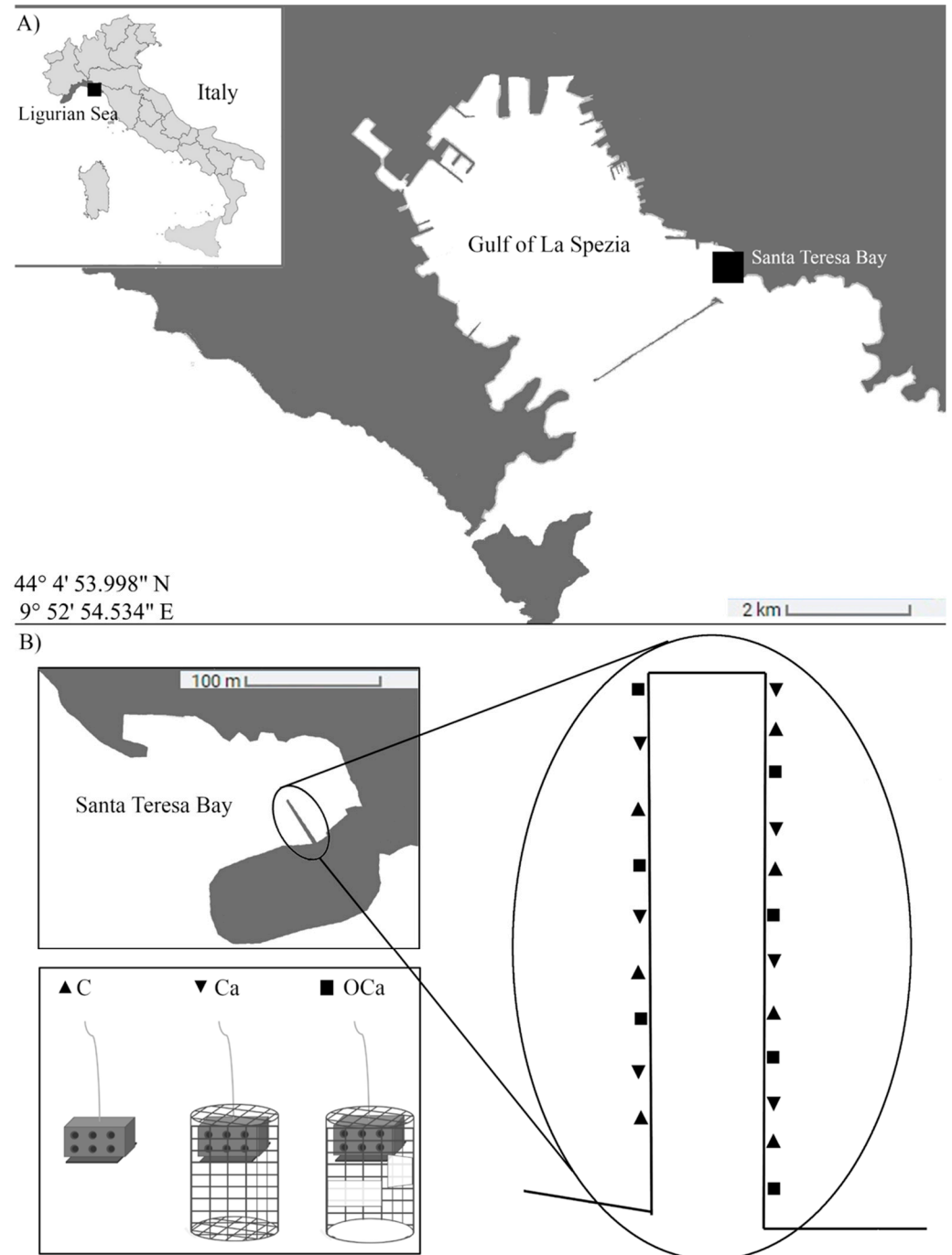


Figure 1. (A) General map of the location of the study area within the Gulf of La Spezia, Western Mediterranean Sea, Ligurian Sea, Italy. (B) Map of Santa Teresa Bay with the scheme of the different treatment levels of the experimental units deployed along the floating pontoon. The symbols indicate the treatment levels deployed with a scheme of the experimental units used: C, control; Ca, caged; and OCa, open-caged PVC panels.

The experiment was conducted in late summer, which can still be considered a favorable season for fouling growth in the study area, as highlighted in previous monitoring of NIS in fouling communities carried out in the Gulf of La Spezia in 2018, including the bay of Santa Teresa [41].

2.2. Experimental Design

The experimental units were 14×14 cm PVC panels attached to the downward face of bricks, in order to keep the panels down at a depth of about 1 m and to limit the algal colonization by hanging them from the floating pontoon. The experimental unit followed the structure proposed by the Smithsonian Environmental Research Center to collect fouling communities, which has been used since 2018 in the Gulf of La Spezia as a continuous monitoring program, including in the bay of Santa Teresa [41]. The present manipulative experiment added the use of cages to exclude macro-predators. The experimental treatment comprised three levels with seven replicates each: control (C), open-caged (OCa) and caged (Ca) PVC panels. Two panels (one belonging to C and one to Ca) were lost due to external causes out of the 21 units deployed. Cages were green plastic cylinders, about 25 cm in diameter and 50 cm in height, with a mesh size of about 0.5 cm, fixed to bricks with plastic cable ties. The treatment level OCa was included as a control for potential artifacts induced by the experimental manipulation, (i.e., the presence of cages on some panels) [42], and was composed of a cage opened at the bottom and with two additional holes of about 30×30 cm disposed at different heights along the cage, in order to remove about half of the cage surface (Figure 1B). The replicates of the treatment levels were distributed along the floating pontoon, alternating them consequently (Figure 1B).

2.3. Field Measurements

A GoPro[®] camera was deployed underwater during cage cleaning days and during the retrieval of panels, for about four hours in the late morning, in order to qualitatively evaluate the presence and activity of predatory macrofauna through time-lapse photography (one photo every 30 s). Furthermore, any cases of predation on fouling communities were recorded during deployment and retrieval of the experimental units, as well as during cage cleaning days.

All the cages were manually cleaned with brushes 28 and 55 days after deployment, in order to allow water circulation inside. At the end of the experiment, all panels were retrieved, weighted, photographed (Supplementary Figures S1–S3) and preserved in plastic bags with 70% ethanol.

2.4. Laboratory Analyses

In the laboratory, all panels were observed under dissecting microscopes for taxonomic identification of sessile invertebrates, obtaining a complete list of species per panel. For each panel, wet weight (estimating the biomass (B, in grams)), species richness (S) and coverage (C, as a percentage) of each taxon were calculated, considering only sessile macrozoobenthos. B was measured to assess if predation could have an influence on the total biomass of assemblages. Specifically, the B value was obtained by weighting the wet panels as soon as they were retrieved, then letting them drain for 5 s. The collected samples were carefully analyzed in order to obtain a list of species for each panel (S) and a biogeographic status was assigned to each taxon (*sensu* Chapman and Carlton [43]), namely NIS and native species. Cryptogenic species, (i.e., species that cannot be considered either native or introduced due to lack of information) were conservatively included in the group of native species for the statistical univariate analyses, while unresolved taxa (species identified at a higher taxonomic level due to insufficient resolution, e.g., damaged specimens or juveniles) were excluded. Cryptogenic and unresolved taxa were about 13 and 7%, respectively, of the total taxa recorded. Finally, the coverage of the whole fouling assemblage was assessed by the point count method ($n = 50$ points), that allows the multiple count of different taxa on the same point, (i.e., the total coverage can exceed 100%) [44].

2.5. Statistical Analyses

Differences in the mean values of B (wet weight), species richness of native species and NIS (S_{native} and S_{NIS}), Shannon Index (H' based on $\log e$) and coverage of native species and NIS (C_{native} and C_{NIS}) were assessed by one-way ANOVA tests (fixed factor: “treatment level”, with three levels: C, OCa, Ca; unequal sample size). The differences among treatment levels were also assessed for abundance (mean coverage among treatment levels > 1%). In all cases of non-homogeneity of the variances, data were properly transformed. Tukey’s HSD tests were calculated for post-hoc comparisons.

Principal Coordinate Analysis (PCO) among samples (square-root transformation; resemblance matrix obtained using the Bray–Curtis similarity index) was used to visualize the pattern of fouling communities of experimental panels. The PERMDISP test and one-way PERMANOVA [45] were carried out to test differences within and between levels of factor “treatment level” (fixed, three levels: C, OCa, Ca). Moreover, SIMPER analyses showing the contribution of NIS to the dissimilarities among experimental conditions were performed. All the analyses were carried out with the software Microsoft Excel, R [46] and PRIMER 6 with the PERMANOVA+ add-on package [45,47].

3. Results

3.1. Presence of Predators

A total of 395 photos, taken automatically under the floating pontoon, were obtained during the experiment, but only two (native) fish species of potential macro predators were identified: the Osteichthyes *Oblada melanura* (Linnaeus, 1758) and *Sarpa salpa* (Linnaeus, 1758). Moreover, during sampling and management procedures, the sea urchin *Arbacia lixula* (Linnaeus, 1758) was found grazing on a panel, and predation by *O. melanura*, *S. salpa* and *Chromis chromis* (Linnaeus, 1758) on fouling communities colonizing the floating pontoon was noted by snorkeling operators.

3.2. Sessile Fauna on Panels

A total of 46 sessile taxa (including 10 NIS) belonging to seven taxonomic groups was found on all panels, independently from the experimental treatment level (Supplementary Table S1). All the NIS observed had already been recorded in the Gulf of La Spezia, with the exception of *Amphibalanus eburneus* (Gould, 1841), which was absent in the yearly monitoring of the same year carried out in Santa Teresa [41] but was present in this experiment. The structure of the fouling communities in the different treatment levels showed a dominance of calcareous species (especially bryozoans and serpulids, i.e., *Salmacina* sp. and *Schizoporella errata* (Waters, 1878)) in C and OCa panels, while Ca panels were abundant in “soft” species.

The univariate tests revealed only two significant differences, for H' ($F = 5.54$, degrees of freedom: 2, $p = 0.01$) and C_{native} ($F = 4.11$, degrees of freedom: 2, $p = 0.04$). In particular, H' was higher in Ca than in C (adjusted p -value (p') = 0.025) and in OCa ($p' = 0.029$); while mean C_{native} was significantly lower in Ca than in C ($p' = 0.03$), but not significantly lower than in OCa ($p' = 0.26$) (Figure 2; Supplementary Table S2).

Differences in mean C_{NIS} (those with a total mean coverage above 1%) were tested among treatment levels, namely for *Branchiomma* sp., *Branchiomma luctuosum* (Grube, 1870), *Hydroides elegans* (Haswell, 1883) and *Styela plicata* (Lesueur, 1823). The results of the ANOVA tests are reported in Table 1. *Branchiomma luctuosum* coverage (Figure 3) was significantly different among treatment levels (Table 1), with a higher value in Ca than in C (p -adj = 0.004) and in OCa (p -adj = 0.04). Also *S. plicata* coverage (Figure 4) was significantly different among treatment levels (Table 1), with a lower value in Ca than in OCa (p -adj = 0.03), but no significative difference was highlighted between C and Ca panels (p -adj = 0.13).

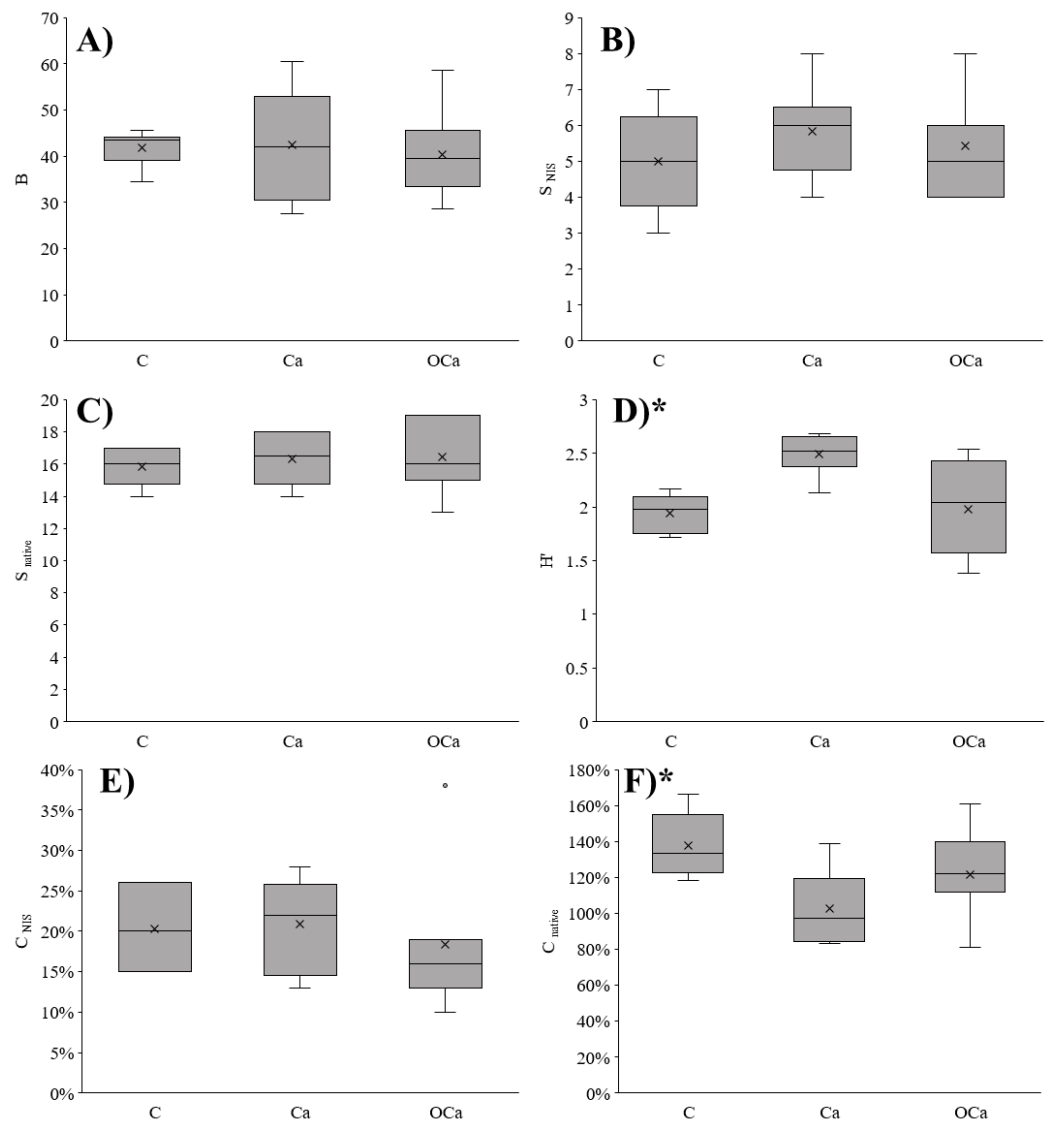


Figure 2. Boxplots of: (A) panel wet weight (B; g); (B); NIS richness (S_{NIS}); (C) native species richness (including cryptogenic species; S_{native}); (D) Shannon Index (H'); (E); NIS coverage (C_{NIS} ; %); (F); native species coverage (C_{native} ; %). Data were grouped by experimental treatment levels: C: control panels, (i.e., uncaged); OCa: open-caged panels, (i.e., artifact control); Ca: caged panels. “×” in the plots indicates mean values. Core lines show median values, the box size is based on the interquartile ranges and whiskers show minimum and maximum values. Significant variables are marked with an asterisk (*).

Table 1. Summary of the ANOVA tests on mean coverage of selected NIS (with total mean coverage among all plates above 1%). °: ANOVA test on square root- transformed data. Significant p values are in bold.

NIS (Mean Coverage > 1%)	Mean Coverage	F Value	p
<i>Branchiommma</i> sp.	2.8%	0.4316	0.6568
<i>Branchiommma luctuosum</i> (Grube, 1870)°	3.2%	7.7513	0.0044
<i>Hydroides elegans</i> (Haswell, 1883)	9.7%	2.1297	0.1513
<i>Styela plicata</i> (Lesueur, 1823)	1.5%	4.4003	0.0300

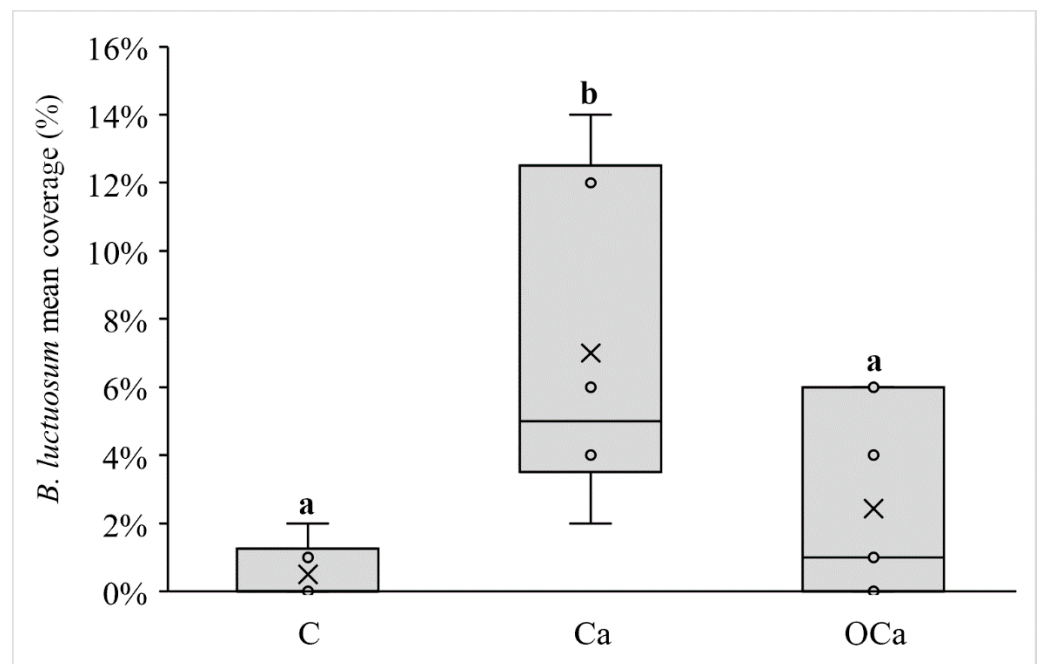


Figure 3. Boxplots of *B. luctuosum* coverage area on panels of the three experimental conditions. “x” in the plots indicates mean values. C: control panels, (i.e., uncaged); OCa: open-caged panels, (i.e., artifact control); Ca: caged panels. Significant differences among treatment levels ($p < 0.05$) are indicated by letters.

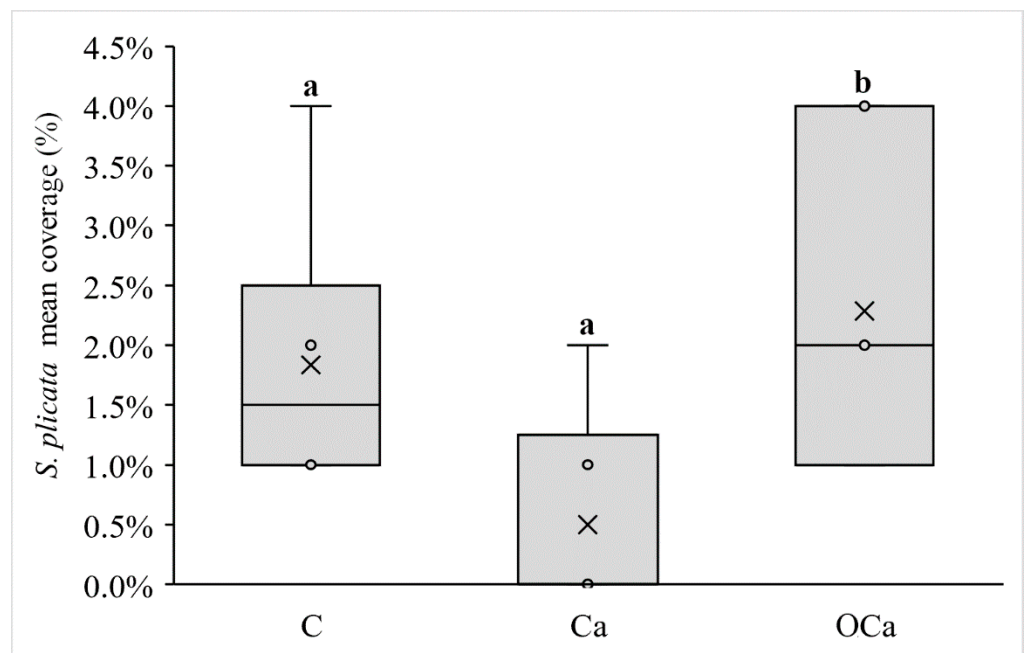


Figure 4. Boxplots of *S. plicata* coverage area on panels of the three experimental conditions. “x” in the plots indicates mean values. C: control panels, (i.e., uncaged); OCa: open-caged panels, (i.e., artifact control); Ca: caged panels. Significant differences among treatment levels ($p < 0.05$) are indicated by letters.

Regarding multivariate analyses, PCO showed high similarity in fouling assemblages of the same treatment level and a clear separation among assemblages of different treatment levels, mostly along the first axis. As expected, C and OCa assemblages resulted closer to each other than C and Ca samples (Figure 5). Taxa vectors most correlated to the ordination

(Pearson’s $R > 0.7$), correspond to the native species *Anomia ephippium* Linnaeus, 1758, *Salmacina* sp., *S. errata* and *Simplaria* sp., and to the NIS *A. eburneus* (Figure 5).

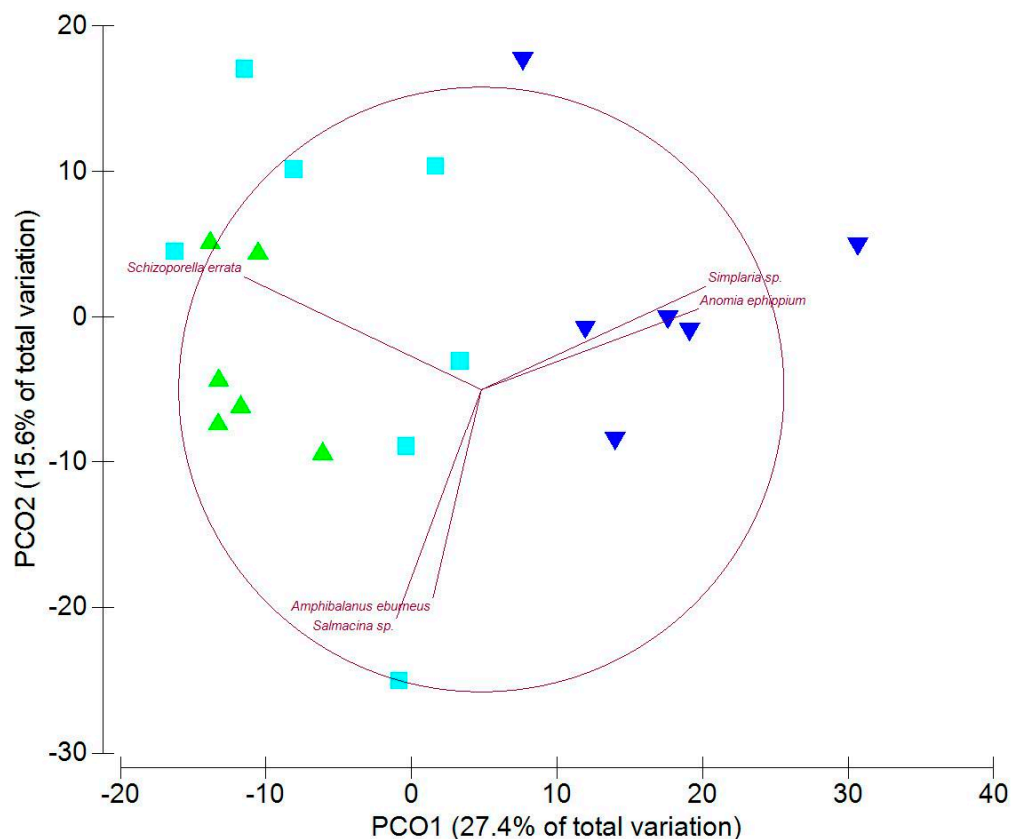


Figure 5. PCO on the Bray–Curtis similarity index (square root transformed data) among the panels of C, Ca and OCa treatment levels. Vectors of correlated taxa (≥ 0.7) are shown. C: control panels, (i.e., uncaged; green triangle); OCa: open-caged panels, (i.e., artifact control; blue triangle); Ca: caged panels (light blue square).

The PERMDISP test showed homogeneity of multivariate dispersion ($F = 2.2, p = 0.23$), while the PERMANOVA main test showed a significant difference among assemblages from different treatment levels (Pseudo- $F = 2.70; p = 0.0004$; Unique perm = 9918). In particular, post-hoc pairwise tests based on Monte Carlo simulations were performed due to few possible unique permutation outcomes and showed a difference in the fouling assemblages between C and Ca ($t = 2.17; p = 0.002$), and OCa and Ca ($t = 1.60; p = 0.03$). No difference was highlighted between C and OCa assemblages ($t = 0.90; p = 0.55$).

Finally, SIMPER analysis revealed the contribution of single taxa to the similarity within treatment levels and to the dissimilarity among them (Supplementary Tables S3 and S4). More in detail, assemblages from the C treatment level showed the highest average similarity (71.57%). The NIS contributing most to this similarity were *H. elegans* in C and in OCa, while it was *B. luctuosum* in Ca (Supplementary Table S3). In terms of native taxa contribution, *S. errata* was the species contributing most in all treatment levels, followed by *Salmacina* sp. in C and OCa, and *A. ephippium* in Ca (Supplementary Table S3). The highest average dissimilarity among treatment levels was observed between C and Ca (42.28%; Supplementary Table S4). Native and non-indigenous species contributing most to dissimilarity were *B. luctuosum* and *S. errata* for C–Ca and Ca–OCa, and *H. elegans* and *Salmacina* sp. for C–OCa (Supplementary Table S4).

4. Discussion and Conclusions

An effect of predation exclusion on the overall structure of fouling assemblages under the different experimental treatment levels was assumed with multivariate analyses. As far as the experimental set up used, the control of the artifact (OCa) revealed no effects of the cages, other than the exclusion of predators. Considering the univariate analyses, only two variables showed significantly different results among treatment levels. In particular, H' and C_{native} were higher in caged panels, suggesting that these results can be ascribed to the absence of predation, since predators could not act as regulators of community dynamics after being excluded by the cages [48]. The replication of the experiment in other localities or the deployment of more replicates could possibly detect the predation effect with the other selected variables. On the other hand, the lack of significant differences among treatment levels for the rest of the observed variables may be interpreted as no-effect of predation on the fouling organisms growing on the panels, although the observable low power of the tests (due to the high p -values obtained) cannot grant a statistical significance [49].

A significant effect of predation was observed when exploring the individual responses of two NIS, *B. luctuosum* and *S. plicata*, although showing opposite patterns. *Branchiomma luctuosum*, originally described in the Red Sea [50], was first recorded in the Gulf of La Spezia in 2018 [41] and in Santa Teresa Bay showed a relatively high abundance under the floating pontoon (personal observations of the authors). *Styela plicata* is a NIS from the northwestern Pacific region [51], known in the Gulf since 2010 and recorded in Santa Teresa during the NIS monitoring carried out at the site in 2018 [41,52]. In our manipulative experiment, *B. luctuosum* was indeed more abundant in the caged panels, while *S. plicata* was more abundant in the not-caged panels. These responses suggest that predators are likely to control the development of the two species and may regulate interspecific competition for settlement space. In particular, *B. luctuosum* was likely affected by predation, while *S. plicata* was not strongly influenced by predation, probably due to its structural characteristics and chemical deterrents [53], but it may lose competition with other species when the assemblage is not exposed to predators. Therefore, on the basis of our observations, the prevalence of *S. plicata* in a fouling assemblage could be favored by the presence of predators. Similarly, Gauff et al. [54] found an undisturbed growth of the congeneric *Styela clava* Herdman, 1881 under predation effect. Osman and Whitlatch also made similar observations [12] on *Didemnum* sp., as well as Needles et al. [55] on a different target species, the encrusting bryozoan *Watersipora subtorquata* (d'Orbigny, 1852). On the contrary, the major reduction of surface coverage of *B. luctuosum* on uncaged panels supports the hypothesis that predation can limit the success of NIS, as observed in other studies [3,16,56].

The information on the presence and activity of predator organisms in the experimental site are only anecdotal and the sampling effort was limited to short time frames. During visits to the site, the photographic records and the snorkeling sessions identified three common fish species around the suspended experimental gear and predating/grazing fouling assemblages in the bay. *Oblada melanura* is very common in the Mediterranean Sea and can be considered an opportunistic predator [57,58]. Its diet depends on both season and specimen size, and can include crustaceans, copepods and several benthic and pelagic species. *Sarpa salpa* is generally considered an herbivorous fish [59], but juveniles also eat crustaceans, including copepods [60]. Its role in the predation of fouling communities can therefore have more effect on algae rather than on macroinvertebrates. *Chromis chromis* is a common species living both in rocky shores and in artificial reefs. It feeds on small planktonic or benthic animals [61], so it can be included in the list of potential predators. It was not possible to record any other predator interacting with the experimental systems, except for a specimen of sea urchin found once on a panel, i.e., *A. lixula*. The assessment of the predatory activity during the sampling procedures could have acted as a source of disturbance and benthic predators could have interacted with panels during the night or in periods other than those recorded. An integration of the ecological experiments with new tools, such as the remote video foraging system proposed by Chebaane et al. [6] or the

tethering approach using target fouling species [62], should be considered in the future, in order to directly evaluate which species actually forage or graze on the experimental units.

As far as the effect of predation against colonization by NIS (biotic resistance), the results of this experiment are not conclusive, in particular regarding the observation of opposite effects in two NIS known to be well-established in the study area. Different predation effects have been described when considering different taxa [63,64]. An experiment on fouling communities in the tropical eastern Atlantic also revealed different trends, defining the biological interactions as an intricate network of processes [40]. Despite the potential of native predators to eventually provide resistance to NIS, it is unlikely that native fouling communities could fully resist NIS introductions. Nevertheless, the preservation of healthy native predator populations can find application in fouling management. The use of benthic predators and grazers as “natural antifouling agents” has been suggested in order to contrast fouling growth in particular contexts, such as aquaculture cages or touristic marinas [30,65].

In conclusion, this experiment can be considered as a preliminary study in the assessment of predation effects on fouling communities. This approach should be replicated, as this topic is still completely unexplored in other Mediterranean localities. The strength of predation pressure in this basin should be compared with that shown in tropical areas and at higher latitudes, adding new data for the assessment of the consumptive biotic resistance hypothesis. In fact, the high species diversity in the Mediterranean, compared to that of other temperate regions, might offer an interesting complement to evaluate the hypothesis of predation as a driver of diversity [33], which is still an open question in the literature.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/jmse10101496/s1>: Supplementary Figures S1–S3; Tables S1–S4. Figure S1: Pictures of two PVC panels pertaining to the treatment level “control panels”; Figure S2: Pictures of two PVC panels pertaining to the treatment level “open-caged panels”; Figure S3: Pictures of two PVC panels pertaining to the treatment level “caged panels”; Table S1: List of taxa found in the panels of predation exclusion experiment. NIS are in bold; Table S2: Summary of the ANOVA tests on the response variables. Significant p values are in bold; Table S3: SIMPER table showing taxa contribution to dissimilarity within treatments in the experiment. Data were square-root transformed, and Bray-Curtis index was used to calculate dissimilarity. Av.Abund: average abundance; Av.Diss: average dissimilarity; Sim/SD: ratio between similarity and standard deviation. Contrib%: single taxon contribution; Cum.%: cumulative percentage of contributions. NIS are shown in bold. C: control panels; Ca: caged panels; OCa: open-caged panels; Table S4: SIMPER table showing taxa contribution to dissimilarity between treatments in the experiment of aim 3. Data were square-root transformed, and Bray-Curtis index was used to calculate dissimilarity. Av.Abund: average abundance; Av.Diss: average dissimilarity; Diss/SD: ratio between dissimilarity and standard deviation. Contrib%: single taxon contribution; Cum.%: cumulative percentage of contributions. NIS are shown in bold. C: control panels; Ca: caged panels; OCa: open-caged panels.

Author Contributions: M.T., J.F. and A.O.-A. conceived the study. M.T., J.F. and L.P. performed the field work and laboratory analyses. M.T. and L.P. carried out the statistics. M.T. wrote the first draft of the manuscript with the contribution of J.F. and A.O.-A. acquired funds. All authors have read and agreed to the published version of the manuscript.

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