Point pattern analysis as a tool for assessing disease spread and population features in remaining sanctuaries of the critically endangered bivalve *Pinna nobilis*

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Running tittle: Population condition in *Pinna nobilis*

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ABSTRACT

An emergent disease has relegated populations of the Mediterranean pen shell, *Pinna nobilis* L. critically endangered to sanctuaries featuring salinities outside the 36.5 to 39 range. Point pattern analysis was used in three areas of the Alfacs Bay (Ebro Delta) still hosting pen shells to assess the possible undergoing of disease spread by comparing the spatial distribution of live individuals vs. empty shells across spatial scales. We also evaluated the importance of other ecological aspects of conservation relevance such as the size distribution of individuals, and the possible association to seagrass habitats. The population assessment showed no recent mortality and a clear dominance of large adults among empty shells (97.3%) pointing to no disease spread during the study period. At the low spatial scale Nearest Neighbor (NN) analyses evidenced significant clustering (NN Ratios of 0.4-0.8), but in one of the zones NN distances were closer in empty shells than in live individuals, suggesting a former localized outbreak. At the larger spatial scale, MDSCA confirmed clustering patterns up to distances of 115 to 190 m, with higher aggregation of empty shells at the same study zone. The bay also featured low juvenile availability (3.2%), which risks the continuity of the population. No evidence for habitat or conspecific selection could be observed from abundance patterns and variation in NN across study regions. Our research provides a tool for assessing population condition in paralic environments, where salinity conditions tend to slow down disease spread, thus allowing a time gap for undertaking conservation decisions.

Keywords: spatial distribution; Nearest Neighbor Analysis; Multi-distance Spatial Cluster Analysis; size class structure; population condition

1. Introduction

The fan mussel, *Pinna nobilis*, is an endemic Mediterranean bivalve currently listed as a "critically endangered" species by the Spanish government (BOE 251–14181, 2018), and the International Union for Conservation of Nature (IUCN) (Kersting et al., 2019). In the fall of 2016, mass mortality events (MMEs) close to 100% of the individuals started to occur in southern Spanish waters and have spread to the rest of the Mediterranean Sea in less than three years (García-March et al., 2020b). The etiological agent responsible for MMEs was first identified as the protozoan *Haplosporidium pinnae* (Catanese et al., 2018), although synergistic effects with a new species of *Mycobacterium* (Carella et al., 2019; 2020; Šarić et al., 2020) and/or Vibriosis (Rodríguez et al., 2018, Prado et al., 2020b, Künili et al., 2021; Lattos et al., 2021) cannot be discarded. At present, only isolated populations in reservoirs such as coastal bays and lagoons featuring salinity conditions frequently outside the 36.5 to 39 range appear to experience lower shellfish mortality rates associated to the disease (e.g., Cabanellas-Reboredo et al., 2019; Prado et al., 2021; Katsanevakis et al., 2021) and may act as larval sources for a future recovery of the species. However, due to their semi-closed regimes, these areas are subjected to large environmental variations, which may bring them close to the optimum conditions for the disease. Having a tool to detect the spread of mortalities and simultaneously assess local population dynamics, is fundamental for the long-term conservation of populations.

The spatial arrangement of benthonic communities is decided in the moment of settlement and metamorphosis as well as by benthic processes occurring at local spatial scales (Prado et al., 2012). Gregarious settlement usually occurs in response to an attractive substance or stimuli that is associated with the presence of a suitable habitat or conspecifics (Woodin 1986; Kingsford et al., 2002). Further patterns of aggregation may also arise from rejection of some types of discrete substrates because of the presence of negative cues including surface texture and conspecifics pheromones as observed for the barnacle *Balanus improvisus* (Berntsson et

al., 2004). Larval settlement may be also affected by passive factors such as depth (Prado et al., 2020a) since shallow areas constitute a physical barrier that can alter the direction or speed of currents. Spatial patterns of populations are expected to reflect larval settlement selection and a combination of passive transport patterns and larval planktonic behavior (Hoffmann et al., 2012). Such patterns may be obscured by early benthic processes such as physical disturbance, predation, and competition and/or physiological stress and disease which may cause large mortalities of over 90% of all juveniles (Hunt & Scheibling, 1997; Gosselin & Qian, 1997). Yet, mapping and imaging techniques are still considered to provide a valuable 'footprint' of factors determining settlement patterns in sessile organisms (Gosselin & Qian, 1997).

Spatial pattern analysis is a widely used technique in the field of forest ecology to understand propagule dispersal and vegetative reproduction processes that characterize the life strategy and colonization success of plant species (Hamill and Wright, 1986; Little and Dale, 1999). It has been successfully used to assess the effects of intra- and interspecific competition, the spread of contagious diseases, or growth facilitation by other species in wild vegetal populations (Kenkel, 1988; He & Duncan, 2000; Rejmánek & Lepš, 1996; Haase et al., 1996; Moeur, 1997; Gienke et al., 2014; Maggi et al., 2017). For instance, Kenkel (1988) examined the positions of live and dead trees and found that the distribution of live trees was locally highly regular indicative of an inhibition distance, while the dead trees were significantly more clumped than random mortality would dictate. Haase et al., (1996) observed significantly clumped patterns resulting from a strong association with other persistent plant species whereas seedlings germinating under the canopy of conspecifics tended to die within days or weeks. Among distance-based methods, the cumulative distribution function given by the distance to the nearest neighbor (Clark & Evans, 1954) and the Ripley's *K* function (Ripley, 1977) are two of the most widely used approaches for mapped data (see reviews by Liu, 2001; Dixon, 2002). The nearest neighbor analysis is useful for assessing processes occurring at the small spatial scale but does not allow detecting distances between aggregates whereas the *K*

97 function allows for the analysis of patterns at a hierarchy of scales, including the distances
98 between aggregates, since it is based on the distances between each point and all the other
99 points in the pattern (Rozas & Camarero, 2005). Potentially, these tools could be also applied
100 to study the spatial arrangement of sessile marine invertebrates after pelagic dispersion from
101 adult sources as well as for detecting possible infection outbreaks by comparing aggregation
102 patterns of living versus dead individuals. However, to our knowledge, this is a disregarded
103 approach in the marine environment, possibly because it is not always possible to obtain a
104 point map of the target species distribution and alternative methods such as quadrates and
105 transects have prevailed (e.g., Basso et al., 2015; Cox et al., 2017).
106 Spatial patterns of distribution may be more easily obtained for large sessile species such as
107 *Pinna nobilis* thus providing valuable information about the possible influence habitat features
108 and/or the presence of conspecifics in spatial patterns of distribution. Pen shell MMEs are
109 caused by a disease that appears to be very highly specific and infective in open waters
110 (García-March et al., 2020b). However, infection rates can be considerably decreased in paralic
111 environments with salinities outside the 36.5 to 39 range, particularly in non-summer periods
112 (Prado et al., 2021) and are therefore expected to reflect foci of infection and the potential
113 spread of the pathogen(s) when comparing point patterns of live individuals vs. empty shells.
114 Other monitoring techniques such as PCR are so far of limited efficiency because unequivocal
115 diagnosis requires the sacrifice of the animal for a biopsy of the digestive gland (Mihaljević et
116 al., 2021), and post-mortem evaluation is also complicated by rapid decomposition of tissues
117 in seawater (Brooks 2016, Huang et al., 2021). Besides, this technique alone also fails in
118 providing spatial patterns of disease spread, which is highly necessary for decision makers
119 prior to the implementation of possible conservation actions such as localized translocation of
120 remaining animals, or freshwater treatments to maintain salinities below the 36.5 threshold
121 (Cabanellas-Reboredo et al., 2018; Prado et al., 2021).

In this context, three different areas of the Alfacs Bay still featuring important numbers of individuals were evaluated with the following objectives: (1) to assess the occurrence of possible disease outbreaks by comparing patterns of spatial distribution in live individuals vs. empty shells in terms of density and dispersion (i.e., regular, random or clumped) across spatial scales ranging from 10s to 100s of m using spatial analysis techniques of point patterns; (2) to evaluate the size distribution of individuals (juveniles, young adults, and adults) as a proxy of the frequency of recruitment events needed for the persistence of the species, and estimate age-dependent mortality; and (3) to assess abundance patterns by habitat type and depth in order to identify possible habitats of conservation interest. In addition, we provide an updated evaluation of the status of remaining populations of *P. nobilis* in different areas of the Alfacs Bay, one of the last sanctuaries of species in the Western Mediterranean.

2. Materials and Methods

2.1. Study areas

Alfacs Bay is a semi-enclosed estuarine water body with an approximate surface of 49 km^2 and a maximum depth of ca. 6 m. It is located in the southern part of the Ebro River Delta (Catalonia, NW Mediterranean), and has a great economic importance due to the presence of extensive shellfish aquaculture, particularly racks of Pacific oyster and Mediterranean mussel along its northern coast. The study was conducted in three zones of the bay that still host populations of *Pinna nobilis* (Prado et al., 2020a; Prado et al., 2021): i) Zone 1 is in the middle region of the Banya Peninsula; ii) Zone 2 in the far end of the Trabucador sand bar; and iii) Zone 3 is in the north coast of the Alfacs Bay, adjacent to the St. Joan Tower (Fig. 1).

Zones 1 and 2 are located within the Natura 2000 network area, with pen shell individuals distributed along the shallow sandbar (ca. 0 to 130 cm depth) that constitute the ancient coastline of the delta and features meadows of the seagrass *Cymodocea nodosa* (Ucria) Asch., and/or beds the green macroalgae *Caulerpa prolifera* (Forsskål) J.V.Lamouroux, 1809 on a sand

148 bed (Prado et al., 2014). The pen shell parasite *H. pinnae* was detected in few individuals in
149 Zone 1 in 2018 and 2020, whereas *Mycobacteria* sp. seems to be absent in middle and inner
150 regions of the Bay (Prado et al., 2021). However, no previous information on pathogen's
151 presence is specifically available for Zones 2 and 3. No MMEs have been reported in these
152 zones, and the occurrence of the parasite is expected to decrease closer to the Trabucador
153 Sand Bar due to slightly lower salinities (Prado et al., 2021). Zone 3 is located immediately in
154 front of rice field drainage channels that seasonally release freshwater high in nutrients and
155 organic matter that favors the development of epiphytes and fast growing macroalgae (Prado,
156 2018) but also causes the reduction in salinity that appears to be protecting pen shells from
157 MMEs (Prado et al., 2021). During preliminary surveys conducted in summer 2020, no
158 evidence of disease was observed in this region of the bay (P. Prado, personal observation).
159 The area hosts *Zostera noltei* in shallow silty and muddy areas closer to the freshwater
160 discharge, while deeper areas (up to ca. 200 cm) are fully dominated by *C. prolifera* with some
161 patches of *C. nodosa* (Prado, 2018).
162
163 *2.2. Field sampling*
164 Measures of salinity and temperature were conducted weekly in the different study zones
165 using a YSI 6660 multiparametric probe equipped with a 650 MDS data logger in order to
166 capture as much as possible temporal variability in these variables that could influence disease
167 outbreaks. A total of 4.1 ha were sampled in Zone 1 (Banya Peninsula), 5.8 ha in Zone 2
168 (Trabucador), and 4.2 ha in Zone 3 (St. Joan Tower), depending on local working conditions and
169 available human resources to conduct the search. In zones 1 and 2, water transparency and
170 shallow depth allowed for a direct visual identification of pen shells and recorded features by
171 observers across previously marked areas. Sampling in these two zones was usually conducted
172 parallel to the line of the shallow sandbar. In zone 3, featuring greater depth and lower
173 visibility conditions in most of the area, the work was conducted with the support of a snorkel

174 team that placed a position mark next to each individual for later georeferencing. Monthly
175 salinity values were measured during the study period at each zone.
176 Pen shells were georeferenced from March to early July 2021 (a 4-month sampling period
177 was used because previous local research has shown that mortality in the Alfacs Bay is driven
178 by salinity patterns and not by differences in sea temperature; Prado et al., 2021) using a Leica
179 Zeno FLX100 Smart Antenna attached to a 2-m carbon fiber pole that allowed marking a
180 position in shallow water. The antenna was also connected to a Leica Zeno Tab 2 Rugged
181 Android Tablet featuring the Leica Zeno Connect application for the transmission of high
182 precision (2 cm) GNSS positions from the antenna. For each pen shell encountered, we marked
183 the condition (live individuals or empty shells), noting also recent mortality that can be
184 determined by the absence of fouling in the inner part of the shell. Since the Alfacs Bay is a low
185 hydrodynamics area, empty shells may remain in their position for many years and provide
186 knowledge about historic patterns of abundance, as well as information on mortality resulting
187 from recent contagious diseases.
188 Animals were measured in width and their total length estimated based on a previously
189 stablished relationship for the local population ($y = 0.207x + 7.16$; $R^2 = 0.718$; $p < 0.0001$).
190 Resulting estimates were grouped into three classes to determine size structure: i) The
191 smallest class consisted of large juveniles of ca. $20 \leq x < 30$ cm; ii) medium class, young adults of
192 intermediate size ($30 \leq x < 50$ cm), and iii) large adults ≥ 50 cm, which in the Alfacs Bay may
193 attain maximum ages of ca. 7-9 years (Prado et al., 2020a). Also, depth and habitat type
194 including seagrass meadow (*C. nodosa* and *Z. noltei*), turf of macroalgae (*C. prolifera*) or mixed
195 beds and sandy areas were recorded. Data were downloaded into a laptop for further data
196 analyses with different GIS software, depending on specific needs (see below).

197

198 *2.3. Data analyses*

199 The totality of the surface covered in each zone (each one with different dimensions and
200 shape) was first split into equal units of 1,000 m^2, using the divide/ modify feature into
201 proportional parts tool available in ArcGIS Pro 10.7.1. This yielded a total of 41 units in Zone 1,
202 58 units in Zone 2, and 42 units in Zone 3. For each zone and unit, we calculated the number of
203 live, empty shells, and total individuals of each size, as well the number of individuals of each
204 condition within each habitat using the count point features within polygon features tool in
205 QGIS Desktop 3.18.2.
206 Differences in the number of individuals by size class (juveniles, young adults, and adults)
207 among study zones was investigated with separate one-way MANOVAs for live individuals,
208 empty shells, and total counts. Further one-way MANOVAs were also used to assess zone
209 differences in the number of individuals within habitats (seagrass, *Caulerpa*, mixed vegetation,
210 and sand) for each condition and total shells. Differences among zones in the number of live
211 individuals, empty shells, and total shells were also investigated with one-way ANOVA.
212 For all MANOVAs and ANOVAs, normality (Chi-square test) and homogeneity of variances
213 (Cochran's test) were tested, and when necessary, data were log transformed to meet these
214 assumptions. All analyses were performed using Statistica v.12 software.
215
216 *2.4. Spatial analysis techniques of point patterns*
217 The average nearest neighbor (ANN) and Multi-distance Spatial Cluster Analyses (MDSCA)
218 available in ArcGIS 10.7.1 were used to investigate the type of point pattern distribution
219 (random, clumped, or regular) of live individuals, empty shells, and total shells at each study
220 area and to assess the size of potential clumps. The ANN has been proposed to be more
221 adequate at the low spatial scale (< 20 m; Rozas & Camarero, 2005), whereas MDSCA provides
222 more informative results at greater distances (up to 100s of m).

223 The ANN is given as the ratio between the observed mean distance between each feature
224 and its nearest neighbor ($\bar{D}o$) and the expected mean distance for features given in a random
225 pattern ($\bar{D}e$):

226 $$ANN = \frac{\bar{D}o}{\bar{D}e} = \frac{\sum_{i=1}^{n} d_i / n}{0.5 \Big/ \sqrt{n/A}} \qquad (1)$$

227 where d_i is the distance between the feature point *i* and its nearest neighboring feature, *n*
228 corresponds to the total number of features, and *A* is the total surveyed area specified by the
229 user and the polygon feature.

230 The average nearest neighbor z-score value for the statistic level (*p* value) at which
231 observed pattern deviated from expected random pattern was calculated as:

232 $$z = \frac{\bar{D}o - \bar{D}e}{SE} \qquad (2)$$

233 Where:

234 $$SE = \frac{0.26136}{\sqrt{n^2/A}} \qquad (3)$$

235

236 MDSCA in ArcGIS is a transformation of the original Ripley's *K* Function which is named as
237 *L(d)*:

238 $$L(d) = \sqrt{\frac{A \sum_{i=1}^{n} \sum_{j=1, j \neq 1}^{n} K_{i,j}}{\pi n (n-1)}} \qquad (4)$$

239 where *d* is the distance, *n* is the total number of point features in the study area feature (*A*),
240 and $K_{i,j}$ is a weight. Depth was used as a weight field in the model in order to account for
241 possible effects in larval settlement. Also, given the pen shell distribution along the shallow
242 sandbars of the ancient coastline, the edge correction method was used to correct for possible
243 underestimations near the edge of the study area.

244 Given the large number of available point features (see next section), a total of 99
245 permutations was used to run the model and generate higher and lower confidence envelops.

246 We used the default program setting including 10 distance bands, with 1 m as initial distance
247 and increments of 50 m, and the user provided study area feature class available in the tool.
248
249 **3. Results**
250 *3.1. Monthly records of salinity and temperature*
251 Monthly averages of salinity during the study (March to July 2021) varied between 35.7 ±
252 0.2 and 36.1 ± 0.4 in the Banya zone (Zone 1) and between 35.6 ± 0.2 and 36.2 ± 0.3 in the
253 Trabucador (Zone 2). However, salinities above 36.5 (i.e., the lower limit for parasite infection;
254 Cabanellas-Reboredo et al., 2019) were detected during two consecutive weeks in the Banya
255 and once in the Trabucador zone, in late July. In the St. Joan Tower (Zone 3), mean monthly
256 salinities were lower than in outer zones, with values between 34.7 ± 0.7 and 35.7 ± 0.2 during
257 the study period, with values always below 36.5.
258 For temperature, monthly averages increased from 14.7 ± 0.05 to 26.5 ± 0.4 °C in the Banya
259 zone (Zone 1), from 14.6 ± 0.03 to 26.8 ± 0.8 °C in the Trabucador (Zone 2), and from 15.3 ±
260 0.04 to 27.2 ± 0.7 °C in the St. Joan Tower (Zone 3) between March and July 2021.
261
262 *3.2. Abundances across study sites*
263 In the Banya Peninsula (Zone 1) a total of 3,137 shells were found within the study area of
264 4,1 ha. Out of these, 45.9% of individuals were live individuals and the remaining 54.1% were
265 empty shells (Fig. 2). Among live individuals the majority (81.5%) were large adults, with a
266 lesser component of young adults (17.1%) and even lower component of juveniles (1.4%). In
267 the Trabucador (Zone 2), 1,651 shells were recorded within a larger area of 5.7 ha, although
268 with a similar proportion of live individuals vs. empty shells (51.7 and 48.3%, respectively). The
269 bulk of the live population were adults (45.3%), and young adults (46.8%), and there was also a
270 larger component of juveniles than in Zone 1 (7.9%). The St. Joan tower area (Zone 3) hosted
271 the lowest number of shells (650 pen shells in 4.2 ha), although with a higher proportion of live

individuals vs. empty shells (62.2 vs. 37.8%) (Fig. 2). In this zone, live individuals were dominated by young adults (60.6%), followed by adults (39.4%), with no observation of juveniles (only 1 individual was found at a very shallow depth but was outside the study area).

MANOVA analyses with the size structure of individuals confirmed the presence of significant differences among the three study areas, consistently for live individuals, empty shells, and total shells (Table 1A-C; Fig. 3A-C). The Banya zone showed the highest abundance of live individuals, empty shells, and total shells, followed by the Trabucador, and the St. Joan Tower zone, with patterns being mostly driven by a greater abundance of large adults. For live individuals and total shells, young adults in the Banya and Trabucador zones showed similarly higher abundances than the St. Joan Tower zone, while juveniles were significantly higher in the Trabucador, and lower in the Banya, and the St. Joan Tower zone, where none was observed. In contrast, differences in the abundance of empty shells were entirely due to the presence of large adults (Table 1B, Fig. 3B), which comprised 99.5, 95.6, and 93.5% of empty shells in the Banya, Trabucador, and St. Joan Tower zones, respectively. Only 23 young adults and 2 juvenile empty shells were found in the Banya zone, 30 young adults and 5 juveniles in the Trabucador, and 14 young adults and 1 juvenile in the St. Joan Tower zone. Among observed empty shells, only 2 adults, 1 young adult and 1 juvenile in the Trabucador were recent.

MANOVA results with habitat groups also evidenced the presence of further significant differences among sites (Table 1D-F; Fig. 3D-F). The Banya zone hosted most of live individuals and empty shells, which were significantly more abundant in unvegetated sandy areas (83% and 77.6% for live individuals and empty shells, respectively); this habitat being comparatively scarce in the other two zones. The seagrass *C. nodosa* hosted most of the remaining individuals in the Banya zone (16.8 and 22.3% of live individuals and empty shells), and similar to slightly lower abundances were observed for individuals in the Trabucador. The St. Joan Tower zone hosted the highest abundance of individuals in *C. prolifera* (82.1 and 71.1% for live individuals

and empty shells), followed by the Trabucador (26.6 and 20.1%, respectively), and negligible in the investigated area of the Banya Peninsula. The Trabucador zone featured higher abundances of individuals in mixed habitats of *C. nodosa* and *C. prolifera* (45.4 and 47.6% for live individuals and empty shells), followed in much lower abundance by the St. Joan Tower zone (7.4 and 4.9%, respectively).

One-way ANOVA results for live individuals, empty shells and total shells confirmed overall patterns, with higher abundances in the Banya zone, followed by the Trabucador, and lowest in the St. Joan Tower zone (Table 1G-I; Fig. 3A-C).

The depth distribution of individuals also displayed notable differences among sites (Fig. 4A-C). In the Banya Peninsula, individuals were found at depths ranging from 20 to 115 cm, peaking at 60 to 80 cm for both live individuals and empty shells, and similar ranges (10 to 125 cm) could be also found in the Traducador, although with a broader peak (40 to 80 cm). In contrast, the St. Joan Tower zone features a deeper distribution, peaking at 120 to 130 cm and still high abundances of individuals at 175 cm with an unknown depth limit outside the study area.

3.3. Point patterns analyses

Results for the Nearest Neighbor (NN) analyses showed significant clustering patterns (values < 1) in all study zones (Table 2). Clustering was slightly higher in the Trabucador (Zone 2) than in the central part of the Banya Peninsula (Zone 1), and lowest in the St. Joan Tower area (Zone 3) which showed the highest NN Ratios and Z Scores, particularly for empty shells. There was also a remarkable similarity in the NN ratios of live individuals vs. empty shells evidencing the persistence of spatial patterns over large periods of time. In two of the study zones, the Banya Peninsula, and the St. Joan Tower observed NN distances were either equal or larger than those observed for empty shells, respectively (Table 2). In contrast, in the Trabucador zone NN distances were slightly closer for empty shells than for live individuals.

Results for Multi-distance Spatial Cluster Analyses (MDSCA) provided additional information about the size of the aggregations at greater spatial scales (100s of m). In the Banya Peninsula (Zone 1), animals were clustered up to distances of 125 m (live animals), 115 (empty shells), and 120 m (combined data) when all shells were included in the analysis. In the Trabucador (Zone 2), clustering patterns reached larger distances ranging from 175 m (live animals) to up to 190 m (empty shells), and 180 m for combined data. Intermediate values were obtained for the St. Joan Tower area (Zone 3), where pen shells we clustered until distances of 170 m (live animals), 140 (empty shells), and 160 m (combined data). Beyond those distances, MDSPA results evidenced tendency to dispersion. In Zone 1, the significance of these patterns with respect to the higher and lower confidence envelopes was very little (Fig. 5A-C). In Zone 2, pen shells showed significant clustering at distances below 100 to 130 m, and significantly dispersed at distances from ca. 180-190 to 320-360 m in all groups (live individuals, empty shells, and both combined) (Fig. 5D-F). In contrast, Zone 3, consistently displayed significant clustering at distances up to ca. 140 to 170 for all groups (live individuals, empty shells, and combined data) but patterns of dispersion were non-significant at greater distances (Fig. 5G-I).

4. Discussion

Our results show that the three study areas of the Alfacs Bay still host (by July 2021) a large abundance of individuals –a total of 2,697 pen shells found alive in 14 Ha– that constitute one of the few remaining reservoirs of the species in the Mediterranean, and the largest sanctuary within Spanish waters. Our findings are comparable to the 2,762 live individuals observed during 258 visual surveys throughout Greece by 9 research groups, most of them in the Kalloni Gulf (Lesvos Island) and in Laganas Bay (Zakynthos Island) (Zotou et al., 2020). Yet, the total size of populations within remaining areas is still unknown. The Alfacs Bay spreads over an estimated area of ca. 49 km^2, but the outer half of the bay was severely impacted by the disease in 2018 (Prado et al., 2021). Besides, the local pen shell distribution seems very

restricted to shallow areas, mostly from ca. 20 to 130 cm of water depth, excepting in areas of the North coast, where it can reach over 175 cm of water (Prado et al., 2014; this study). In the absence of an accurate bathymetry, we roughly estimate that ca. 500 ha of suitable bay habitat might remain to be surveyed for population abundance. The three study zones presented distinctive densities of live individuals ranging from 1,440 (3.5 ind./ 100 m^2) in the Banya, 853 (1.5 ind./ 100 m^2) in the Trabucador, and 404 (0.96 ind./ 100 m^2) in the St. Joan Tower zone, which appear to be related to local habitat quality and historical patterns of recruitment success (Prado et al., 2014, 2020a). The Northern coast of the Alfacs Bay is immediately adjacent to drainage channels from extensive rice agriculture and reveals signs of siltation and eutrophication in submerged vegetation (Prado, 2018), that might have negative effects on respiration and feeding of benthic invertebrates (Thorson, 1950). In fact, the bathymetric distribution in the St. Joan Tower zone appears to be related to distance from silt deposit areas as reported elsewhere (Katsanevakis, 2006). In contrast, the Banya Peninsula is located further away from the source of anthropic influence and closer from the mouth of the bay region which features a more reduced residence time of water (ca. 5-10 days; Cerralbo et al., 2019) that allowed connectivity with other populations previously to MMEs (Wesselmann et al., 2018). Despite such less favorable effects of habitat quality, paradoxically, the proximity to freshwater discharges provides enhanced protection against the advance of pen shell disease. Prado et al., (2021) monitored cumulative pen shell mortality in three sites of the Banya Peninsula and found a positive association with the salinity gradient (37.4 to 35.7), with higher rates close to the mouth of the bay (100% of individuals) and intermediate (43%) to low rates (13%) in the middle and inner regions of the Banya Peninsula. During the study (March to early July 2021) monthly averages of salinity were below the 36.5 low range limit established for disease transmission (Cabanellas-Reboredo et al., 2019), although with exceptions during late July (after georeferencing sampling) in the Banya zone (2 consecutive weeks) and in the Trabucador (1 week), that greatly increase infection risk.

The dynamics of disease acquisition and transmission in other organisms such as grapevine *Vitis vinifera* infected with "flavescence dorée" has evidenced variations in clustering patterns that were attributed to the temporal spread of the epidemics (Maggi et al., 2017). Also, Gienke et al., (2014), used point pattern analysis to examine the effect of beech bark disease and reported enhanced severity – i.e., a clustering effect– in beech saplings located in the proximity (< 5 m) of highly cankered canopy beech trees. In contrast, our results for NN analysis showed a general similarity in patterns of aggregation between live individuals and empty shells, that coupled with scarce recent mortality in all zones (0.15% of total empty shells) points to a disease-free scenario within study zones. Yet, slightly lower NN ratios in empty shells occurred in the Trabucador zone (i.e., higher clustering) resulting in lower NN distances which suggest the trace of a previous outbreak. In fact, the area was directly connected with the open sea during a ca. 4-month period due to breakage of the Trabucador sand bar during the Gloria Storm from January to May 2020 (Pintó i Fusalba et al., 2020). Samples from five juveniles found by fishermen in the outer sea just in front of the Trabucador sand bar during that period were all found to be positive to the parasite by PCR (G. Catanese, pers. comm.) and reinforce the possible entrance of the disease and a subsequent stabilization. Besides, MDSCA for the Trabucador also evidenced clustering effects at greater distances in empty shells than in live individuals (190 vs. 175 m) further supporting the occurrence of previous disease effects. Both NN and MDSCA showed enhanced clumping of hosts in the Trabucador zone, which be a relevant factor limiting the advance of a disease vector during the initial phase of an epidemic process (Caraco et al., 2001). Conversely, both the Banya Peninsula and the St. Joan Tower showed greater larger aggregation in live individuals (by 10 and 30 m, respectively). For the Banya Peninsula this apparent absence of disease effects contrast with the 43% mortality observed in middle regions by Prado et al., (2021). Yet, monitoring circles were located ca. 250 m beyond the end of the assessed area, pointing that this middle zone of the bay marks the transition from more affected to less

affected areas. Besides, given the large dominance of large adults in the area, an effect of increased natural mortality cannot be discarded given the low age expectancy of Alfacs Bay individuals (dominance of the 8 ± 1 year-old class and a maximum age of 15 years; Prado et al., 2020a). In fact, the abundance of empty shells (ca. 54.1%, 48.3%, and 37.8% of the total shells observed in the Banya, Trabucador, and St. Joan Tower zones, respectively) was completely biased towards large adults, which comprised up to 97.3% of the total, with only a minor component of young adults and juveniles (2.4 and 0.3%, respectively). Since that the Banya zone hosted a size distribution with a greater component of adults than the other investigated zones (90.7% vs. 70.1 and 60%), higher mortality could be mostly attributed to the presence of older size classes. Alternatively, an enhanced disappearance of younger, brittle shells, could also be possible, but is not supported by recruitment surveys and annual deployment of collectors in the Alfacs Bay from 2016 (Prado et al., 2019; Kersting et al., 2020).

The relative similarity in MDSCA clustering patterns across the three study zones featuring contrasting types of dominant benthic habitats (sand in the Banya zone, mixed vegetation in the Trabucador, and *Caulerpa* in the St. Joan Tower), also suggest the absence of preferential habitat settlement, as reported for some species of bivalves such as the Northern quahog *Mercenaria mercenaria* (Bachelet et al., 1992) or the Mediterranean mussel *Mytilus galloprovincialis* (Cáceres-Martínez et al., 1994). Effects of differential habitat mortality are considered unlikely due to considerably higher presence of individuals in bare sandy patches of the Banya Peninsula, which would have facilitated access to local predators such as decapods, cephalopods and fishes, and the gastropod *Hexaples trunculus*, at lengths < 45 cm (refuge size) compared to enhanced protection provided by vegetated areas (Farina et al., 2009, García-March et al., 2007; Kersting & García-March, 2017). Alternatively, they could be the combined result of a limited dispersal capacity of oocytes and larvae from the emission source, local circulation, and coastal geomorphology features. Oocytes of *P. nobilis* have been indicated a low buoyancy and a tendency to sink (Trigos et al., 2018), and the mobility of the D-larvae also

428 appears to be lower than in other bivalves (P. Prado, personal observ.), which might constrict
429 dispersal in confined waters. Besides, according to Cerralbo et al., (2019), water residence time
430 in middle to inner parts of the Alfacs Bay embracing the three study zones ranges from 35 to
431 45 days; a considerably longer period than the expected planktonic duration of *P. nobilis* larvae
432 (Trigos et al., 2018) and may favor local recruitment in favorable years. Another important
433 factor responsible for aggregation appear to be the presence of very shallow sand bars –
434 remains of the ancient coastline–, acting as a physical barrier for larval dispersal towards the
435 coast (Prado et al., 2020a). Their presence explains the distribution of pen shells along belt
436 areas parallel to shore and the tendency towards disperse patterns at larger scales in all zones.
437 Compared to other bivalves where clustering in dense matts or reefs are the result of selective
438 settlement patterns (Grassle et al., 1992; Vasquez et al., 2013), those of the pen shell appear
439 to be due to an early life-stage strategy based on low buoyancy and passive drift. The possible
440 effect of parental detection in larval settlement cannot be fully discarded, but there was
441 considerable variability in mean NN distances to another shell across zones (from only 1.2 in
442 the Banya to 3.1 m in the St. Joan Tower), which suggest that adult proximity is not a necessary
443 requirement.
444 The age structure of the live pen shell population in the Alfacs Bay was dominated by adult
445 and young adult individuals, although with significant differences among study sites. Older
446 individuals with greater size were dominant in the Banya Peninsula (8.15%), whereas in the
447 Trabucador and the St. Joan Tower, there were increasing numbers of young adults (46.8 vs.
448 45.2% and 60.6 vs. 39.3%, respectively for each age size and zone), evidencing that
449 recruitment events are sparse and not homogeneous within the bay. The overall juvenile
450 abundance was very low across the different zones, with values close to zero in the St. Joan
451 Tower and the Banya zone (0 to 1.4%) but higher in the Trabucador (7.9%), which is
452 comparable to local spatial variability observed in other studies (e.g., Giménez-Casalduero et
453 al., 2020; Katsanevakis 2006; García-March et al., 2007). Yet, considering the potentially large

size of the remaining population (only discrete bay areas were counted), the presence of juveniles was surprisingly low; to some extent, some individuals could have been overlooked in dense canopy areas but their absence in bare sand areas of the Banya Peninsula where adults were very abundant cannot be misleading. A single pen shell female may release in the order of tens of millions of eggs for reproduction (Trigos et al., 2018), thus supposedly conferring an enormous reproductive potential to the Alfacs Bay population. Conversely, recruitment patterns inferred from shell growth records of the posterior adductor muscle suggest window periods of up to 11 years between successive events (Prado et al., 2020a), which is considerably long compared to the more even size structure usually reported in open water populations (García-March et al., 2020a). The reasons for this mismatch between spawning potential and effective recruitment are yet unclear. In open water populations, predation mortality has been proposed as a key factor explaining differences between larval supply and recruitment patterns before MMEs (Kersting & García-March, 2017). Yet, the Alfacs Bay appears to feature a low availability of pen shell larvae throughout the summer (Andree et al., 2018) and recent research both in lab and in the wild points out to the occurrence of a single, main spawning event during the month of May at temperatures ranging between ca. 19-23 °C (P. Prado, personal observ.). Besides, repeated unsuccessful attempts to close the life cycle in captivity using local individuals suggest the undergoing of other factors. First, Prado et al., (2020a) proposed that the presence of simultaneous hermaphrodism –a condition that has been associated with pollution and environmental stress– could lead to in-breeding depression and potentially reduced fertility. Other possible mechanism could be the occurrence of high local inbreeding causing enhanced number of deleterious mutations and consequently reducing fitness in the offspring of highly fecund marine animals (see Plough, 2016). For instance, recent work in the Pacific oyster, *Crassostrea gigas*, has shown that genetic unviability is a major factor driving early life mortality in hatchery and field environments (Plough et al., 2016), and is currently being investigated for *P. nobilis*.

5. Conclusion

Point pattern analysis provided integrative information on population condition and disease outbreaks at several temporal scales. During the study period, the three study zones displayed an apparent absence of disease symptoms but point pattern analysis (both NN and MDSCA) evidenced enhanced clustering of empty shells in the Trabucador zone, which suggest a previous disease condition and was likely slowed or adjourned after the reconstruction of the Trabucador sandbar and the reestablishment of normal conditions with lower salinity (Prado et al., 2021). Aside the Alfacs Bay, surviving populations are also known to exist in confined waters of the Mar Menor Lagoon in Spain (Giménez-Casalduero et al., 2020), the Diana Lagoon in Corse (Simide et al., 2019), the Salses-Leucate, and Thau lagoons in the Gulf of Lion, France (Peyran et al., 2021), the Venice lagoon in Italy (Russo 2017), the Kalloni Gulf (Lesvos Island), and Laganas Bay (Zakynthos Island) in Greece (Zotou et al., 2020) among other possible locations. Yet, survival might be ephemeral without adequate monitoring and management programs. For instance, one of the last fortresses of the species in the Marmara Sea in Turkey appear to have been recently infected and largely lost over a period of only few months (Cinar et al., 2021a, b), pointing out the need of effective monitoring tools to guide conservation actions such as translocation of animals to aquarium facilities now that robust protocols for captivity maintenance have been developed (Hernandis et al., in prep). Uninfected animals could be also moved to other natural areas or regions with lower infection risk. Besides, in some locations such as the Alfacs Bay, the proximity of agricultural discharge channels could allow the additional release of freshwater during periods of high risk due to increased summer salinities. This action could particularly benefit the conservation of pen shells in middle regions of the Banya Peninsula and the Trabucador, which are sometimes above the lower salinity threshold for parasite infection at 36.5 and might experience an intermittent spread of the disease. Besides, it could also be implemented to preserve estuarine conditions in case of new

breakage of the Trabucador sand bar during heavy storms —such as the Gloria Storm in 2020 (Pintó i Fusalba et al., 2020)—, as foreseen by severe thinning over the last decade. Infection risk also needs to be put in the context of the low presence of juveniles observed in the bay – the last large recruitment was observed in 2017 (Prado et al., 2020a)–, thus making essential the long-term maintenance of populations to allow new recruitment events. There is an imperative need for compromise from the public administrations to start implementing management actions based on proposed scientific evidence to preserve the last remaining sanctuaries of *P. nobilis*.

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524 **References**

525 Andree, K. B., Trigos, S., Vicente, N., Carrasco, N., Carella, F., Prado, P. (2018). Identification of
526 potential recruitment bottlenecks in larval stages of the giant fan mussel *Pinna nobilis* using
527 specific quantitative PCR. *Hydrobiologia*, *818(1)*, 235–247. https://doi.org/10.1007/s10750-
528 018-3616-x

529 Bachelet, G., Butman. C. A., Webb, C. M., Starczak, V. R., Snelgrove, P. V. (1992). Non-selective
530 settlement of *Mercenaria mercenaria* (L) larvae in short-term still-water laboratory
531 experiments. *Journal of Experimental Marine Biology and Ecology, 161*, 241–280.
532 https://doi.org/10.1016/0022-0981(92)90100-O

533 Basso, L., Vázquez-Luis, M., García-March, J. R., Deudero, S. et al. (2015). The pen shell *Pinna*
534 *nobilis*: A review of population status and recommended research priorities in the
535 Mediterranean Sea. *Advances in Marine Biology, 71*, 109–160.
536 https://doi.org/10.1016/bs.amb.2015.06.002

537 Berntsson, K. M., Jonsson, P. R., Larsson, A. I., Holdt, S. (2004). Rejection of unsuitable
538 substrata as a potential driver of aggregated settlement in the barnacle *Balanus improvisus*.
539 *Marine Ecology Progress Series*, *275*, 199-210. http://www.jstor.org/stable/24867679

540 Brooks, J. W. (2016). Postmortem changes in animal carcasses and estimation of the
541 postmortem interval. *Veterinary Pathology, 53* (5), 929–940.
542 https://doi.org/10.1177/0300985816629720

543 Cabanellas-Reboredo, M., Vázquez-Luis, M., Mourre, B., Álvarez, E., et al. (2019). Tracking a
544 mass mortality outbreak of pen shell *Pinna nobilis* populations: a collaborative effort of
545 scientists and citizens. *Scientific Reports, 9*, 1–11. https://doi.org/10.1038/s41598-019-
546 49808-4

547 Cáceres–Martinez, J., Robledo, J. A., Figueras, A. (1994). Settlement and post–larvae behaviour
548 of *Mytitus galtoprovincialis*: field and laboratory experiments. *Marine Ecology Progress*
549 *Series, 112*, 107–117.

Caraco, T., Duryea, M. C., Glavanakov, S., Maniatty, W., Szymanski, B. K. (2001). Host spatial heterogeneity and the spread of vector–borne infection. *Theoretical Population Biology, 59* (3), 185–206. https://doi.org/10.1006/tpbi.2000.1517

Carella, F., Aceto, S., Pollaro, G., Miccio, A., et al. (2019). A mycobacterial disease is associated with the silent mass mortality of the pen shell *Pinna nobilis* along the Tyrrhenian coastline of Italy . *Scientific Reports, 9*, 2725. https://doi.org/10.1038/s41598-018-37217-y

Carella, F., Elisabetta, A., Simone, F., Fulvio, S., et al. (2020). In the wake of the ongoing mass mortality events: Co–occurrence of *Mycobacterium*, *Haplosporidium* and other pathogens in *Pinna nobilis* collected in Italy and Spain Mediterranean Sea. *Frontiers in Marine Science, 7*, 48. doi.org/10.3389/fmars.2020.00048

Catanese, G., Grau, A., Valencia, J. M., Garcia-March, J.R., et al. (2018). *Haplosporidium pinnae* sp. nov., a haplosporidan parasite associated with mass mortalities of the fan mussel *Pinna nobilis* in the Western Mediterranean Sea. *Journal of Invertebrate Pathology, 157*, 9–24. https://doi.org/10.1016/j.jip.2018.07.006

Cerralbo, P., Pedrera Balsells, M. F., Mestres, M., Fernandez, M., et al. (2019). Use of a hydrodynamic model for the management of water renovation in a coastal system. *Ocean Science, 15*, 215–226. https://doi.org/10.5194/os-15-215-201

Clark, P. J., Evans, F. C. (1954). Distance to nearest neighbor as a measure of spatial relationships in populations. *Ecology, 35*, 445–453. https://doi.org/10.2307/1931034

Cinar, M.E., Bilecenoğlu, M., Yokeş, M. B., Güçlüsoy, H. (2021a). *Pinna nobilis* in the south Marmara Islands Sea of Marmara.; it still remains uninfected by the epidemic and acts as egg laying substratum for an alien invader. *Mediterranean Marine Science, 22*, 161–168. https://doi.org/10.12681/mms.25289

Cinar, M. E., Bilecenoğlu, M., Yokeş, M. B., Güçlüsoy, H. (2021b). The last fortress fell: mass mortality of *Pinna nobilis* in the Sea of Marmara. *Mediterranean Marine Science*, 22(3), 669–676. https://doi.org/10.12681/mms.27137

576 Cox, K. D., Black, M. J., Filip, N., Miller, M. R., et al. (2017). Community assessment techniques
577 and the implications for rarefaction and extrapolation with Hill numbers. *Ecology and*
578 *Evolution, 7*, 11213–11226. https://doi.org/10.1002/ece3.3580
579 Dixon, P. M. (2002). Nearest neighbor methods. *Encyclopedia of environmetrics, 3*, 1370–1383.
580 Farina, S., Tomas, F., Prado, P., Romero, J., Alcoverro, T. (2009). Seagrass meadow structure
581 alters interactions between the sea urchin *Paracentrotus lividus* and its predators. *Marine*
582 *Ecology Progress Series,377*, 131–137. https://doi.org/10.3354/meps07692
583 García-March, J.R., García-Carrascosa, A. M., Peña Cantero, A. P., Wang, Y. G. (2007).
584 Population structure mortality and growth of *Pinna nobilis* Linnaeus 1758 Mollusca Bivalvia.
585 at different depths in Moraira bay (Alicante Western Mediterranean). *Marine Biology, 150*,
586 861–871. https://doi.org/10.1007/s00227-006-0386-1
587 García-March, J. R., Hernandis, S., Vázquez-Luis, M., Prado, P., et al. (2020a). Age and growth
588 of the endangered fan mussel *Pinna nobilis* in the western Mediterranean Sea. *Marine*
589 *Environmental Research, 153*, 104795. https://doi.org/10.1016/j.marenvres.2019.104795
590 García-March, J. R., Tena, J., Henandis, S., Vázquez-Luis, M., et al. (2020b). Can we save a
591 marine species affected by a highly infective highly lethal waterborne disease from
592 extinction?. *Biological Conservation, 243*, 108498.
593 https://doi.org/10.1016/j.biocon.2020.108498
594 Giencke, L. M., Dovčiak, M., Mountrakis, G., Cale, J. A., Mitchell, M. J. (2014). Beech bark
595 disease: spatial patterns of thicket formation and disease spread in an aftermath forest in
596 the northeastern United States. *Canadian Journal of Forest Research*, *44(9)*, 1042–1050.
597 https://doi.org/10.1139/cjfr-2014-0038
598 Giménez-Casalduero, F., Gomariz-Castillo, F., Alonso-Sarría, F., Cortés, E., Izquierdo-Muñoz, A.,
599 Ramos, A. (2020). *Pinna nobilis* in the Mar Menor coastal lagoon: a story of colonization and
600 uncertainty. *Marine Ecology Progress Series,652*, 77–94.
601 https://doi.org/10.3354/meps13468

602 Gosselin, L. A., Qian, P. Y. (1997). Juvenile mortality in benthic marine invertebrates. *Marine*
603 *Ecology Progress Series,146*, 265–282. https://doi.org/10.3354/meps146265
604 Grassle, J. P., Snelgrove, P. V., Butman, C. A. (1992). Larval habitat choice in still water and
605 flume flows by the opportunistic bivalve *Mulinia lateralis*. *Netherland Journal of. Sea*
606 *Research, 30*, 33–44. https://doi.org/10.1016/0077-7579(92)90043-E
607 Haase, P., Pugnaire, F. I., Clark, S. C., Incoll, L. D. (1996). Spatial patterns in a two-tiered semi-
608 arid shrubland in southeastern Spain. *Journal of Vegetation Science*, *7(4)*, 527-534.
609 https://doi.org/10.2307/3236301
610 Hamill, D. N., Wright, S. J. (1986). Testing the dispersion of juveniles relative to adults: A new
611 analytic method: Ecological Archives E067-004. *Ecology, 67*, 952–957.
612 https://doi.org/10.2307/1939817
613 He, F., Duncan, R. P. (2000). Density-dependent effects on tree survival in an old-growth
614 Douglas fir forest. *Journal of Ecology, 88*, 676–688. https://doi.org/10.1046/j.1365-
615 2745.2000.00482.x
616 Hoffmann, V., Pfaff, M. C., Branch, G. M. (2012). Spatio–temporal patterns of larval supply and
617 settlement of intertidal invertebrates reflect a combination of passive transport and larval
618 behavior. *Journal of Experimental Marine Biology, and Ecology, 418*, 83–90.
619 https://doi.org/10.1016/j.jembe.2012.03.008
620 Huang, X., Xiong, G., Chen, X., Liu, R., et al. (2021). Autolysis in crustacean tissues after death:
621 A case study using the *Procambarus clarkii* hepatopancreas. *BioMedical Research*
622 International. https://doi.org/101155/2021/2345878
623 Hunt, H. L., Scheibling, R. E. (1997). Role of early post–settlement mortality in recruitment of
624 benthic marine invertebrates. *Marine Ecology Progress Series,155*, 269–301.
625 https://doi.org/10.3354/meps155269
626 Katsanevakis, S. (2006). Population ecology of the endangered fan mussel *Pinna nobilis* in a
627 marine lake. *Marine Ecology Progress Series,1,* 51–59. https://doi.org/10.3354/esr001051

628 Katsanevakis, S., Carella, F., Çinar, M. E., Čižmek, H., et al. (2021). The Fan Mussel *Pinna nobilis*
629 on the brink of extinction in the Mediterranean. *Reference Module in Earth Sciences and*
630 *Environmental Systems*. https://doi.org/10.1016/B978-0-12-821139-7.00070-2
631 Kenkel, N. C. (1988). Pattern of self-thinning in jack pine: testing the random mortality
632 hypothesis. *Ecology, 69*, 1017–1024. https://doi.org/10.2307/1941257
633 Kersting, D., Benabdi, M., Čižmek, H., Grau, A., et al. (2019). *Pinna nobilis*. The IUCN Red List of
634 Threatened Species 2019: e.T160075998A160081499.
635 https://doi.org/10.2305/IUCN.UK.2019-3.RLTS.T160075998A160081499.en
636 Kersting, D. K., García-March, J. R. (2017). Long-term assessment of recruitment early stages
637 and population dynamics of the endangered Mediterranean fan mussel *Pinna nobilis* in the
638 Columbretes Islands (NW Mediterranean). *Marine Environmental Research, 130*, 282–292.
639 https://doi.org/10.1016/j.marenvres.2017.08.007
640 Kersting, D. K., Vázquez-Luis, M., Mourre, B., Belkhamssa, F. Z., et al. (2020). Recruitment
641 disruption and the role of unaffected populations for potential recovery after the *Pinna*
642 *nobilis* mass mortality event. *Frontiers in Marine Science*, 882.
643 https://doi.org/10.3389/fmars.2020.594378
644 Kingsford, M. J., Leis, J. M., Shanks, A., Lindeman, K. C., Morgan, S. G., Pineda, J. (2002).
645 Sensory environments larval abilities and local self–recruitment. *Bulletin of Marine Science,*
646 *70*, 309–340.
647 Künili, İ. E., Ertürk Gürkan, S., Aksu, A., Turgay, E., et al. (2021). Mass mortality in endangered
648 fan mussels *Pinna nobilis* Linnaeus 1758. Caused by co–infection of *Haplosporidium pinnae*
649 and multiple *Vibrio* infection in Çanakkale Strait, Turkey. *Biomarkers*, 1–12.
650 https://doi.org/10.1080/1354750X.2021.1910344
651 Lattos, A., Bitchava, K., Giantsis, I.A., Theodorou, J.A., Batargias, C., Michaelidis, B. (2021). The
652 implication of *Vibrio* bacteria in the winter mortalities of the critically endangered *Pinna*
653 *nobilis*. *Microorganisms, 9*, 922. https://doi.org/10.3390/microorganisms9050922

654 Little, L. R., Dale, M. T. R. (1999). A method for analysing spatio-temporal pattern in plant
655 establishment tested on a *Populus balsamifera* clone. *Journal of Ecology, 87*, 620–627.
656 https://doi.org/10.1046/j.1365-2745.1999.00378.x
657 Liu, C. (2001). A comparison of five distance-based methods for spatial pattern analysis.
658 *Journal of Vegetation Science, 12*, 411–416. https://doi.org/10.2307/3236855
659 Maggi, F., Bosco, D., Galetto, L., Palmano, S., Marzachì, C. (2017). Space–time point pattern
660 analysis of flavescence dorée epidemic in a grapevine field: disease progression and
661 recovery. *Frontiers in Plant Science, 7*, 1987. https://doi.org/10.3389/fpls.2016.01987
662 Mihaljević, Ž., Pavlinec, Ž., Zupičić, I.G., Oraić, D., et al. (2021). Noble Pen Shell (*Pinna nobilis*)
663 Mortalities along the Eastern Adriatic Coast with a study of the spreading velocity. *Journal*
664 *of Marine Science and Engineering, 9* (7), 764. https://doi.org/10.3390/jmse9070764
665 Moeur, M. (1997). Spatial models of competition and gap dynamics in old–growth *Tsuga*
666 *heterophylla*/*Thuja plicata* forests. *Forest Ecology and Management, 94*, 175–186.
667 https://doi.org/10.1016/S0378-1127(96)03976-X
668 Peyran, C., Boissin, E., Morage, T., Nebot-Colomer, E., et al. (2021). Genetic homogeneity of
669 the critically endangered fan mussel *Pinna nobilis* throughout lagoons of the Gulf of Lion
670 (North-Western Mediterranean Sea). *Scientific Reports, 11*, 1–12.
671 https://doi.org/10.1038/s41598-021-87493-4
672 Pintó i Fusalba, J., Garcia-Lozano, C., Sardá Borroy, R., Roig i Munar, F. X., Martí, C. (2020).
673 Efectes del temporal Glòria sobre el litoral. *Treballs de la Societat Catalana de Geografía,*
674 *89*, 89–109. https://doi.org/10.2436/20.3002.01.192
675 Plough, L. V. (2016). Genetic load in marine animals: a review. *Current Zoology, 62*, 567–579.
676 Plough, L. V., Shin, G., Hedgecock, D. (2016). Genetic inviability is a major driver of type III
677 survivorship in experimental families of a highly fecund marine bivalve. *Molecular Ecology,*
678 *25*, 895–910. https://doi.org/10.1093/cz/zow096

679 Prado, P. (2018). Seagrass epiphytic assemblages are strong indicators of agricultural discharge
680 but weak indicators of host features. *Estuarine Coastal and Shelf Science, 204*, 140–148.
681 https://doi.org/10.1016/j.ecss.2018.02.026
682 Prado, P., Andree, K. B., Trigos, S., Carrasco, N., et al. (2020a). Breeding planktonic and
683 settlement factors shape recruitment patterns of one of the last remaining major
684 population of *Pinna nobilis* within Spanish waters. *Hydrobiologia, 847*, 771–786.
685 https://doi.org/10.1007/s10750-019-04137-5
686 Prado, P., Caiola, N., Ibáñez, C. (2014). Habitat use by a large population of *Pinna nobilis* in
687 shallow waters. *Scientia Marina, 78(4)*, 555–565.
688 https://doi.org/10.3989/scimar.04087.03A
689 Prado, P., Carrasco, N., Catanese, G., Grau, A., et al. (2020b). Presence of *Vibrio mediterranei*
690 associated to major mortality in stabled individuals of *Pinna nobilis* L. *Aquaculture*, 519,
691 734899. https://doi.org/10.1016/j.aquaculture.2019.734899
692 Prado, P., Grau, A., Catanese, G., Cabanes, P. et al. (2021). *Pinna nobilis* in suboptimal
693 environments are more tolerant to disease but more vulnerable to severe weather
694 phenomena. *Marine Environmental Research, 163*, 105220.
695 https://doi.org/10.1016/j.marenvres.2020.105220
696 Prado, P., Tomas, F., Pinna, S., Farina, S. et al. (2012). Habitat and scale shape the demographic
697 fate of the keystone sea urchin *Paracentrotus lividus* in Mediterranean macrophyte
698 communities. *PloS one, 7*, e35170. https://doi.org/10.1371/journal.pone.0035170
699 Rejmánek, M., Lepš, J. (1996). Negative associations can reveal interspecific competition and
700 reversal of competitive hierarchies during succession. *Oikos, 76*, 161–168.
701 https://doi.org/10.2307/3545758
702 Ripley, B.D. (1977). Modelling spatial patterns. *Journal of the Royal Statistical Society: Series B*
703 *(Statistical Methodology), 39*, 172–212. https://doi.org/10.1111/j.2517-
704 6161.1977.tb01615.x

Rodríguez, S., Balboa, S., Olveira, G., Montes, J. et al. (2018). First report of mass mortalities in natural population of *Pinna nobilis* A microbial perspective. The 7th Congress of European Microbiologists (FEMS 2018).

Rozas, V., Camarero, J. J. (2005). Técnicas de análisis espacial de patrones de puntos aplicadas en ecología forestal. *Investigación agrária: Sistemas y recursos forestales*, *14(1)*, 79–97.

Russo, P. (2017). Lagoon malacofauna, results of malacological research in the Venice Lagoon. *Bollettino malacologico, 53*, 49–62.

Šarić, T., Župan, I., Aceto, S., Villari, G., Palić, D., De Vico, G., Carella, F. (2020). Epidemiology of noble pen shell (*Pinna nobilis* l. 1758) mass mortality events in adriatic sea is characterised with rapid spreading and acute disease progression. *Pathogens*, *9(10)*, 776. https://doi.org/10.3390/pathogens9100776

Simide, R., Couvray, S., Vicente, N. (2019). Présence de *Pinna nobilis* L 1758. Dans l'étang littoral de Diana Corse. *Marine Life Review, 1–4*.

Thorson, G. (1950) Reproductive and larval ecology of marine bottom invertebrates. *Biological Reviews, 25(1)*, 1–45.

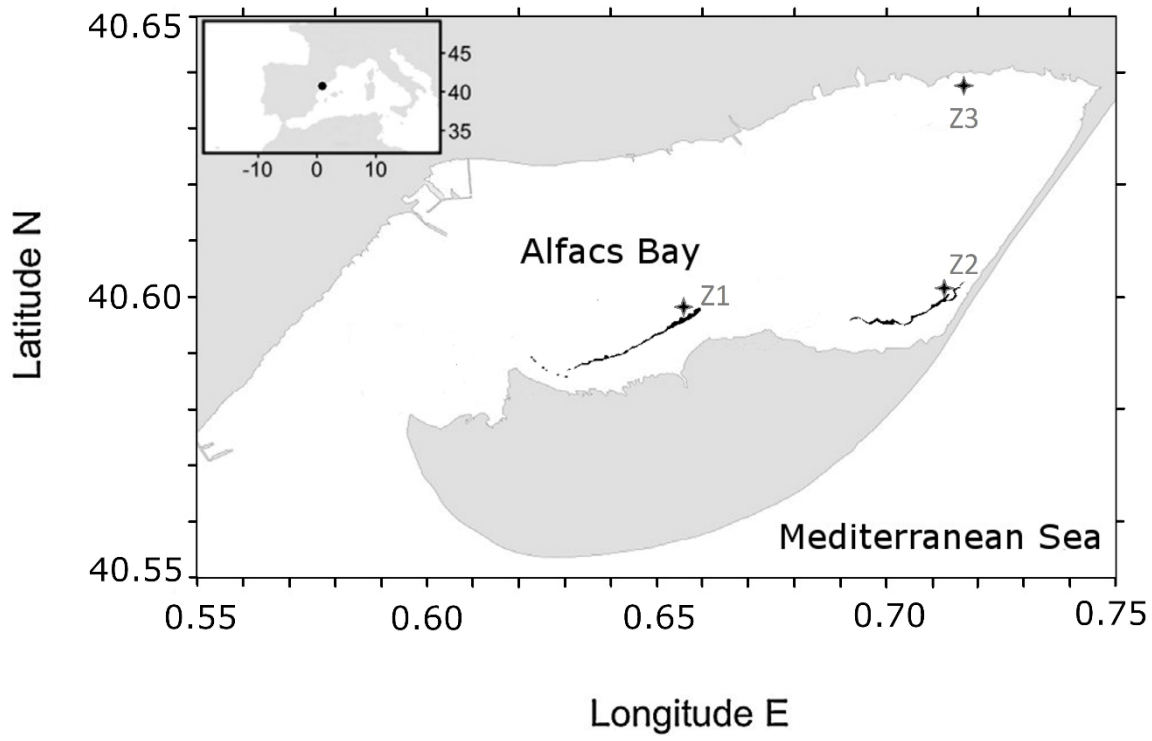
Trigos, S., Vicente, N., Prado, P., Espinós, F. J. (2018). Adult spawning and early larval development of the endangered bivalve *Pinna nobilis*. *Aquaculture, 483*, 102–110. https://doi.org/10.1016/j.aquaculture.2017.10.015

Vasquez, H. E., Hashimoto, K., Yoshida, A., Hara, K., et al. (2013). A glycoprotein in shells of conspecifics induces larval settlement of the Pacific oyster *Crassostrea gigas*. *PloS one, 8*, e82358. https://doi.org/10.1371/journal.pone.0082358

Wesselmann, M., González-Wangüemert, M., Serrão, E. A., et al. (2018). Genetic and oceanographic tools reveal high population connectivity and diversity in the endangered pen shell *Pinna nobilis*. *Scientific reports*, *8(1)*, 1-11. https://doi.org/10.1038/s41598-018-23004-2

730 Woodin, S.A. (1986). Settlement of infauna: larval choice?. *Bulletin of Marine Science, 39,* 401–
731 407.
732 Zotou, M., Gkrantounis, P., Karadimou, E., Tsirintanis, K., et al. (2020). *Pinna nobilis* in the
733 Greek seas NE Mediterranean: on the brink of extinction?. *Mediterranean Marine Science,*
734 *21*, 575–591. https://doi.org/10.12681/mms.23777

735 **Fig. 1.** Map of the Alfacs Bay showing the location of the 3 study areas where live individuals of
736 *P. nobilis* can still be found.
737
738 **Fig. 2.** Maps showing the position of live individuals (green dots) and empty shells (red dots)
739 and the limits of the study area. A) Banya Peninsula (Zone 1); B) Trabucador (Zone 2); and C)
740 St. Joan Tower (Zone 3).
741
742 **Fig. 3.** Pen shell abundances (mean ± SE) within 1,000 m^2 transects at the three study sites. A-
743 C) Size class abundances (adults= A, young adults= YA, and juveniles= J) for live individuals,
744 empty, and total pen shells; and D-F) habitat abundances (sand= SA, Caulerpa= C, seagrass= SE,
745 and mixed vegetation= MV) also for each status and total number of shells.
746
747 **Fig. 4.** Results of MDSCA showing observed vs. expected patterns of clustering and aggregation
748 at increasing distances (for further details see the M and M section) at each study site. A-C)
749 Banya Peninsula: live individuals, empty and total shells, respectively; D-F) Trabucador; and G-
750 I) St. Joan Tower, also respectively for each status and total shells. The high and low
751 confidence envelops are also indicated.
752
753 **Fig. 5.** Depth distribution of live individuals and empty shells by study site. A) Banya Peninsula;
754 B) Trabucador; and C) St. Joan Tower.
755

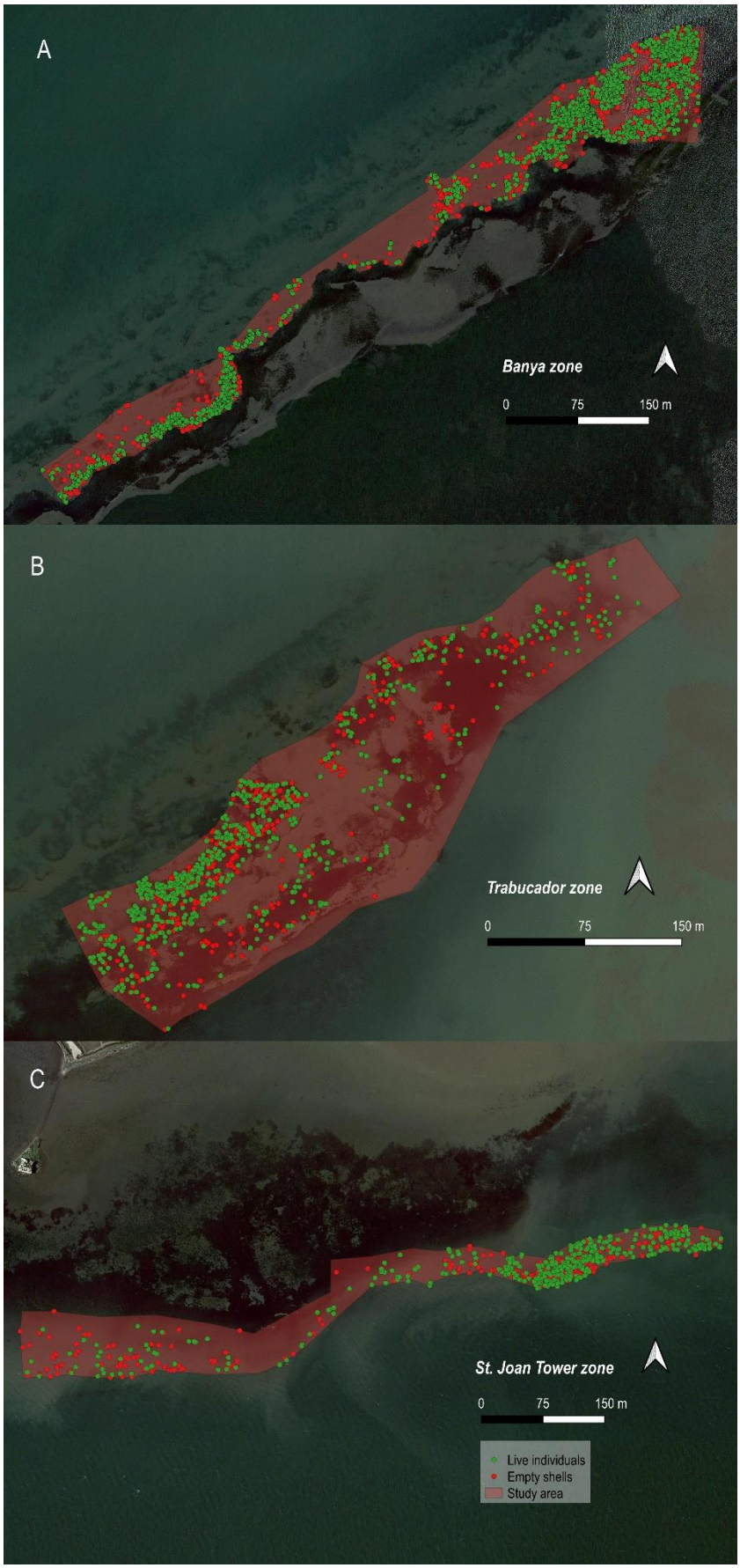


756

757

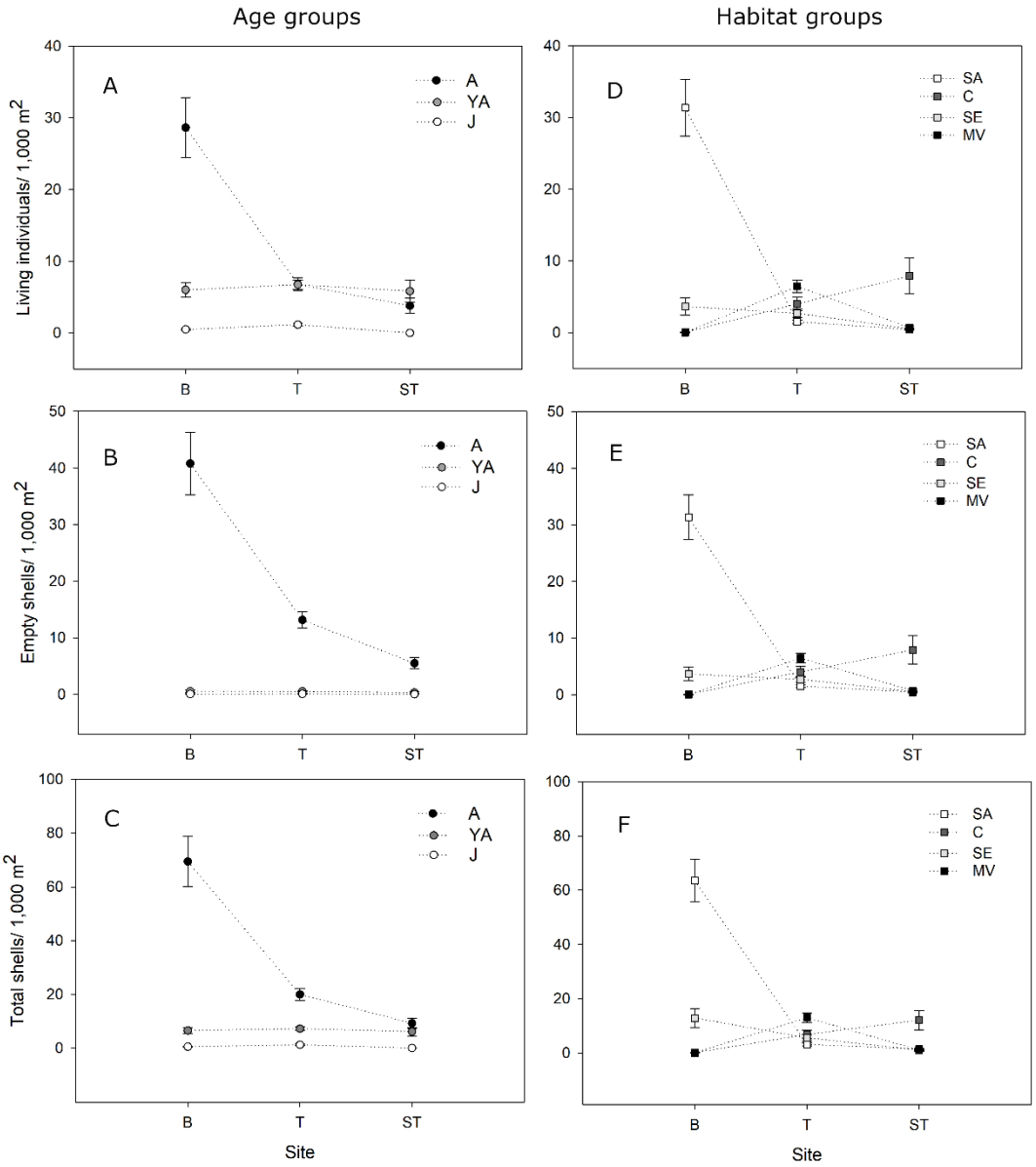
758 Fig. 1.

759


A
Banya zone
0
75
150 m
B
Trabucador zone
0
75
150 m
C
St. Joan Tower zone
0
75
150 m
Live individuals
Empty shells
Study area

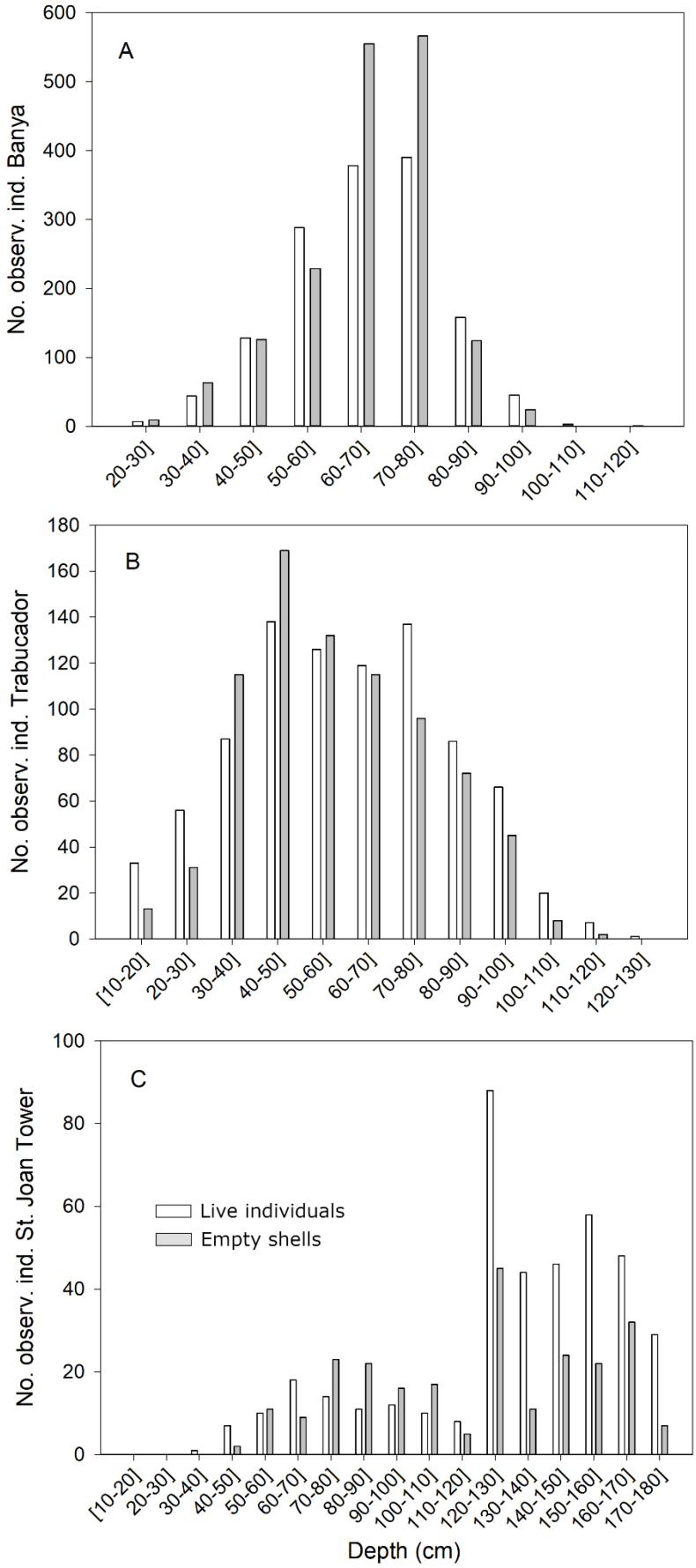
760

761 Fig. 2


Age groups
Habitat groups
A
B
C
D
E
F
A
YA
J
SA
C
SE
MV
Living individuals/ 1,000 m2
Empty shells/ 1,000 m2
Total shells/ 1,000 m2
B
T
ST
Site

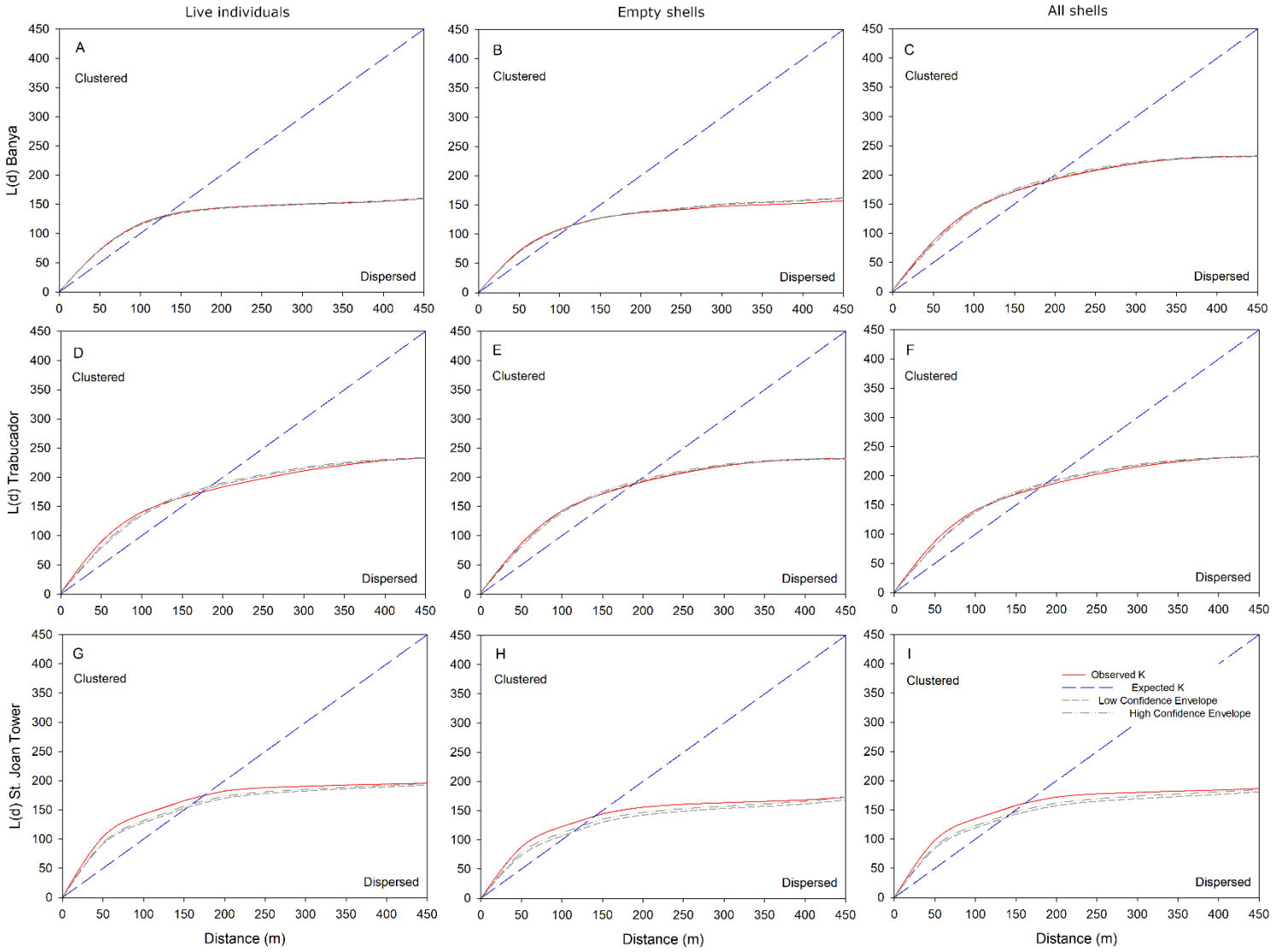
762

763 Fig. 3


A
No. observ. ind. Banya
600
500
400
300
200
100
0
20-30]
30-40]
40-50]
50-60]
60-70]
70-80]
80-90]
90-100]
100-110]
110-120]
B
No. observ. ind. Trabucador
180
160
140
120
100
80
60
40
20
0
[10-20]
20-30]
30-40]
40-50]
50-60]
60-70]
70-80]
80-90]
90-100]
100-110]
110-120]
120-130]
C
No. observ. ind. St. Joan Tower
100
80
60
40
20
0
Live individuals
Empty shells
[10-20]
20-30]
30-40]
40-50]
50-60]
60-70]
70-80]
80-90]
90-100]
100-110]
110-120]
120-130]
130-140]
140-150]
150-160]
160-170]
170-180]
Depth (cm)

764

Fig. 4


Live individuals
Empty shells
All shells
L(d) Banya
L(d) Trabucador
L(d) St. Joan Tower
A
B
C
D
E
F
G
H
I
Clustered
Dispersed
Observed K
Expected K
Low Confidence Envelope
High Confidence Envelope
Distance (m)
0
50
100
150
200
250
300
350
400
450

765 Fig. 5

Table 1. A-C) one-way MANOVA results showing differences among zones (Banya= B, Trabucador= T, and St. Joan Tower= ST) in the age composition (adults= A, young adults= YA, and juveniles= J) of live individuals, empty shells, and total shells; D-F) one-way MANOVAs showing zone differences in habitat distribution (sand= SA, Caulerpa= C, seagrass= SE, and mixed vegetation= MV) for live individuals, empty shells, and total shells; and G-I) one-way ANOVAs for zone differences in the total abundance of live individuals, empty shells, and total shells. For each analysis, significant grouping obtained with SNK post-hoc analyses are shown. Significant values are indicated in **bold**.

MANOVA	A) Live individuals				B) Empty Shells				C) Total shells			
Age groups	Wilk's λ	df	*F*	*P*	Wilk's λ	df	*F*	*P*	Wilk's λ	df	*F*	*P*
Zone	0.573	6, 272	14.50	**0.0000**	0.657	6, 272	10.57	**0.0000**	0.479	6, 272	20.11	**0.0000**
SNK (A)	B> T> ST				B> T> ST				B> T> ST			
SNK (YA)	B= T≥ ST								B= T≥ ST			
SNK (J)	T> B> ST								T> B> ST			
MANOVA	D) Live individuals				E) Empty Shells				F) Total shells			
Habitat groups	Wilk's λ	df	*F*	*P*	Wilk's λ	df	*F*	*P*	Wilk's λ	df	*F*	*P*
Zone	0.174	8, 270	46.93	**0.0000**	0.172	8, 270	47.49	**0.0000**	0.154	8, 270	52.14	**0.0000**
SNK (SA)	B> T> ST				B> T= ST				B> T> ST			
SNK (C)	ST= T> B				ST= T> B				ST= T> B			
SNK (SE)	B= T> ST				B> T> ST				B> T> ST			
SNK (MV)	T> ST= B				T> ST= B				T> ST= B			
ANOVA	G) Live individuals				H) Empty Shells				I) Total shells			
Totals	df	MS	*F*	*P*	df	MS	*F*	*P*	df	MS	*F*	*P*
Zone	2	4.39	21.00	**0.0000**	2	6.30	32.71	**0.0000**	2	5.74	28.75	**0.0000**
Error	138	0.21			138	0.192			138	0.199		
SNK	B> T> ST				B> T> ST				B> T> ST			

783 **Table 2.** Nearest Neighbor (NN) analyses for live individuals, empty shells, and all recorded
784 shells at each study zone. For each analysis, the nearest neighbor index, the Z-Score, the P
785 value, the observed mean distance (m), and the expected mean distance (m) are indicated.
786

	Banya center (Zone 1)			Trabucador (Zone 2)			St. Joan Tower (Zone3)		
	Live	Empty	All	Live	Empty	All	Live	Empty	All
NN Ratio	0.623	0.681	0.670	0.489	0.419	0.450	0.731	0.842	0.788
NN Z-Score	-27.32	-25.08	-35.27	-28.56	-31.38	-42.75	-10.32	-4.71	-10.31
P Value	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
NN Expected	2.667	2.457	1.807	4.118	4.260	2.961	5.101	6.537	4.022
NN Observed	1.663	1.675	1.212	2.014	1.786	1.333	3.732	5.510	3.171

787

788