A multidisciplinary integrated approach using Pachygrapsus marmoratus to assess the impact of port activities on mediterranean marine protected areas

A multidisciplinary integrated approach in *Pachygrapsus marmoratus* to assess the impact of port activities on Mediterranean marine protected areas

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Abbreviations: AChE, acetylcholinesterase activity, Al, aluminum; ANA, acenaphthene; As, arsenic; CAT, catalase; Cd, cadmium, Cr, chromium; Cu, copper; Fe, iron; GR, glutathione reductase; GSH, total glutathione, GST, glutathione S-transferase; Hg, mercury; IDH, isocitrate dehydrogenase; LDH, lactate dehydrogenase; LG, Le Grazie; LPO, lipid peroxidation; Mn, manganese; Ni, nickel; PAHs, polyciclic aromatic hydrocarbons; Pb, lead; PHE, phenanthrene; PM, Porto Mediceo of Livorno; PYR, pyrene; RM, Riomaggiore; SM, Secche della Meloria; TOT HYDR C <10, low molecular weight hydrocarbons (C <10); TOT HYDR C >10, high molecular weight hydrocarbons (C >10); V, vanadium; Zn, zinc

Abstract

The establishment of marine protected areas is considered the main global strategy to halt the loss of marine biodiversity. Since most of marine areas are open systems, this form of habitat protection cannot prevent their contamination due to human activities performed outside of their borders. Innovative approaches to assess the health status of protected marine habitats are therefore needed. Here we developed a multidisciplinary approach that combines ecological, bioaccumulation, cellular and enzymatic markers focused on an intertidal brachyuran crab, *Pachygrasus marmoratus*, to assess the impacts of contaminant exposure on Mediterranean coastal habitats. As study sites we selected two protected areas and two sites within industrial ports of the Ligurian Sea. Our results showed that the selected crab species is an excellent bioindicator. Individuals collected in sites with the highest levels of heavy metal pollution showed the highest signals of stress responses at both cellular and enzymatic levels, coupled with a high incidence of the parasite *Sacculina carcini*, a signal of impairment of their standard development and reproduction cycle. We could also prove that one of the selected marine protected areas showed the same intensity of impact as its adjacent port site. Our multidisciplinary approach proved to be a valuable tool to assess the environmental quality and health of protected and disturbed Mediterranean coastal environments and to inform efficient management and protection schemes for such habitats.

1. Introduction

Marine biodiversity is under a global threat due to several anthropogenic pressures, such as habitat degradation and overexploitation (Webb and Mindel, 2015). There is, therefore, an urgent need to implement new conservation and management measures. Whereas the establishment of marine protected areas (MPAs) is considered the main global strategy for the conservation of marine biodiversity and the restoration of underwater habitats (Grorud-Colvertet et al., 2021), only 6.35% of the world's oceans are designated as MPAs (IUCN, 2017). MPAs were recently also recognised to contribute to the adaptation and mitigation of the impacts of climate change (Simard et al., 2016) and help mitigate other anthropogenic pressures on marine biodiversity, including water pollution from land-based sources (OECD, 2017).

To ensure effective long-term protection of habitats and a spill over effect of protected populations to nearby exploited areas, MPAs cannot simply be areas where a few anthropic activities -usually the transit of boats and fishing- are regulated, but they should be sufficiently large and interconnected to allow the movements of animals and the spread of spores, gametes, and larvae (Roberts et al., 2010). Since most of MPAs are open systems, they are not protected from pollution due to human activities performed outside of their borders (Islam and Tanaka, 2004). This is a critical issue, especially for MPAs that are adjacent to sources of contaminants, such as industrial areas, port zones, mining fields, agricultural and aquaculture farms, urban areas and sewage outfalls. For this reason, it is fundamental to identify and monitor the multiple stressors acting in synchrony and synergically on the protected biota, to estimate the real effectiveness of protected areas in conserving them.

In the last decades, many studies have been published on the effects of pollution on marine organisms, and most of them were based on multi-biomarker approaches (Araujo et al., 2020; Bacchetta et al., 2014; Caricato et al., 2019; Lionetto et al., 2021; Mearns et al., 2019). Recent research highlighted the importance of studying the combined effects of multiple stressors on sentinel

65 organisms through an integrated and multidisciplinary approach that merges ecological, physiological,
66 cellular and molecular studies (Gusso et al., 2015, 2016; Lionetto et al., 2021).

67 Crustaceans, and in particular brachyuran crabs, have often been used to evaluate the
68 ecotoxicological effects of contaminants on estuarine environments (Ben-Khedher et al., 2014; Duarte
69 et al. 2017a; Locatello et al., 2009; Ragunathan, 2017; Rodrigues et al., 2012; 2014), metalliferous
70 waste discharge areas (Tsangaris et al., 2016), mangrove ecosystems (Duarte et al., 2017b; Fusi et al.,
71 2016), coastal areas (Mile and Roster, 1999; Rainbow et al., 2000), and lagoons (Vasanthi et al., 2014).
72 They have also been used as sentinel species in MPA biomonitoring (Araujo et al., 2020; Baratti et al.,
73 2022; Lionetto et al, 2021). To date, however, no ecotoxicological study has integrated ecological,
74 bioaccumulation and biomarker data from crab populations to assess the environmental quality of
75 MPAs.

76 The aim of this study was to develop a novel multidisciplinary approach to assess the
77 effectiveness of MPAs in protecting their coastal habitats from contaminants possibly coming from
78 adjacent harbor areas. To reach this goal we integrated and compared data collected through
79 ecological surveys, chemical analyses, enzymatic tests, and cellular biomarkers on protected and
80 impacted populations of an intertidal organism, ubiquitous on natural shores and artificial structures
81 of the Mediterranean basin, the marbled crab *Pachygrapsus marmoratus*.

82 **2. Materials and methods**

83 *2.1 Study species*

84 The marbled crab *Pachygrapsus marmoratus* (Fabricius, 1787) (Grapsidae; Brachyura;
85 Crustacea) is distributed within the entire Mediterranean basin, the Black Sea, the European and East
86 African Atlantic coasts, the Canary and Azores Islands (Ingle, 1980; Zariquiey Álvarez, 1968). It is a
87 common dweller of the intertidal belt of rocky shores (Cannicci et al., 1999) and it is common on
88 artificial substrata within ports and marinas. This species is resistant to pollution and other stressors
89 and accumulates heavy metals in its tissues (Baratti et al., 2022; Fratini, et al., 2008; Mouneyrac et al.,
90 2001; Shaiek et al., 2018).

91 The adults of *P. marmoratus* are relatively sedentary (Cannicci et al., 1999) and omnivorous,
92 with a diet mainly based on algal and animal preys (Cannicci et al., 2002, 2007). The species breeds
93 from late April to late September (Flores and Paula, 2002; Ingle 1980; Vernet-Cornubert, 1958). The
94 spawning occurs about two weeks after the egg deposition, then the pelagic larvae develop in the
95 water column for about four weeks. The last larval stage, the megalopa, actively swims towards the
96 coastal waters and ultimately recruits and settles in the intertidal habitat, where adults occur.

97 *2.2 Study area and sampling locations*

98 Our study area is the Ligurian Sea, which is the northernmost sector of the western
99 Mediterranean Sea (Cattaneo-Vietti et al., 2010). Within this basin, we sampled two MPAs and two
100 port areas. As MPAs we choose Secche della Meloria (SM), an emerging rocky shoal about 3 miles from
101 Livorno established as a MPA in 2009, and Riomaggiore (RM), a small pebble and rocky shore within
102 the Cinque Terre National Park. As sites representing port areas, we selected the Porto Mediceo of
103 Livorno (PM) and Le Grazie (LG) which are two marinas located within the larger harbor areas of two
104 of the biggest ports of the Ligurian Sea, Livorno and La Spezia, respectively (Fig. 1).

105 *2.3 Pollutant analysis*

106 Levels of inorganic and organic pollutants (trace elements and PAHs) were determined in
107 pools of animals collected during the ecological surveys. A total number of 24 individuals was used.
108 Individuals collected at each site, belonging to both sexes, were pooled into three groups of three
109 animals each. The pools were homogenized using a blender equipped with resin blades and the
110 homogenates were stored at −20 °C until further chemical analyses.

111 <u>2.3.1. Trace element analysis</u>

112 Whole crabs tissue homogenates were dried at 40 °C for 48 h and sieved through a 2 mm mesh
113 size sieve. All analyses were performed according to Baratti et al. (2022). Determinations were
114 validated using NIST SRM 2976 certified material (mussel's tissue). The limit of detection (LOD, *sensu*
115 Sandeep et al., 2018) was 0.3 mg/Kg dry weight for all elements except for Cd (0.03 mg/Kg), Fe (0.8
116 mg/Kg) and Hg (0.0007 mg/Kg). The percent of recovery was >95%.

117 2.3.2. Total PAHs and 16 US-EPA priority PAHs congeners analysis

118 Extraction and GC-MS analysis of PAHs from the whole crab's tissue homogenates were
119 performed as described by Baratti et al. (2022). The limit of detection (LOD) was 0.3 µg/Kg wet weight
120 (w/w). The recovery was > 89%. The analysis of chromatogram was performed using CHROMELEON
121 software (Thermo Fisher Scientific, USA). Measurements were validated using NIST SRM 2974a
122 certified material (mussel's tissue). Additional information was reported in supplementary material.

123 2.3.3. Low Molecular Weight (C<10) and High Molecular Weight (C>10) aliphatic hydrocarbons analysis

124 Aliphatic hydrocarbons represent the most abundant fraction in crude oils and fuels. The
125 extraction of homogenates and the GC analysis of low (C<10) and high (C>10) molecular weight
126 hydrocarbons (C<10) were carried out according with Baratti et al. (2022). For C<10 the LOD was 0.3
127 µg/Kg wet weight, for C>10 the LOD was 1.6 mg/Kg wet weight. The percent of recovery was always
128 over 90%. Additional information was reported as a supplementary material.

129 *2.4 Ecological surveys*

130 2.4.1 Density and population structure

131 At all sites, densities and populations' structure (calculated as sex- and adults/juveniles-ratios)
132 of *P. marmoratus* were assessed through visual censuses and direct sampling surveys (Cannicci et al.,
133 1999) detailed in supplementary material. Surveys were carried out between mid-May and mid-July
134 in 2018 and 2019. At SM, PM and RM the surveys were performed around sunset (i.e., the period of
135 maximum activity of these crabs: Cannicci et al., 1999, 2002), while at LG they were carried out
136 between 11 pm and 2 am, to avoid the high human activity recorded at sunset that impacted the
137 crabs' activities.

138 Crab densities were estimated in two different occasions. Then, for each sampling date, crabs
139 were collected by hand and sexed. Their carapace width, CW, and length, CL, were measured using
140 Vernier callipers (± 0.01 mm). Sex ratio was calculated as no. males / no. total crabs × 100. We also
141 recorded the occurrence of berried females and whether the crabs were parasitized by the cirriped

142 *Sacculina carcini*. The captured crabs were then sacrificed for the biochemical and toxicological
143 analyses.

144 2.4.2 Fertility and fecundity estimation

145 A total of 65 berried females were collected in May-June at all sites. Once captured, females
146 were sedated, placed in individual plastic tubes filled with 80% ethanol and brought to the laboratory
147 of the Department of Biology of the University of Florence. CW and CL of each female were measured
148 using Vernier callipers. Then we counted the number of fertilised eggs carried by each female using
149 the methods described in Simoni et al. (2011) and Cannicci et al. (2011).

150 Following Penha-Lopes et al. (2007, 2009), the average reproductive output of each
151 population was calculated through two ratios: Fecundity per CW ratio (Ratio_Fe), calculated as the
152 number of stage I-II embryos divided by the female CW, and Potential Fertility per CW ratio
153 (Ratio_PoFe), calculated as the number of final stage embryos divided by the female CW. The
154 Ratio_PoFe parameter extrapolates the fertility, i.e., the number of larvae ready to be released by a
155 female (Ford et al., 2003). Additional information was reported as a supplementary material.

156 *2.5 Biomarker analyses*

157 Adult crabs sampled at the four locations were used for biomarker analyses (n =41 from PM;
158 n =19 from SM; n =44 from both LG and RM). From each crab, we collected about 1 mL of haemolymph
159 with a syringe. The digestive gland and two portions of muscle tissues were dissected and stored at -
160 80 °C until further analyses. Neurotoxic effects were evaluated in a muscle portion by AChE activity
161 according to Elman et al. (1961) method. Energy metabolism by IDH and LDH were evaluated in the
162 muscle tissue by Ellis and Golberg (1971), adapted to microplate (Lima et al., 2007) and Vassault (1983)
163 adapted to microplate (Menezes et al., 2006), respectively. Biomarkers of biotransformation,
164 antioxidant defences and oxidative damage were evaluated for the digestive gland by GST (Habig et
165 al., 1974), CAT (Aebi, 1986), GSH (Jollow et al., 1974) and LPO (Bird and Draper 1984, modified, and
166 Ohkawa et al., 1979). Genotoxic effects in haemolymph in terms of micronuclei frequency (MN) and
167 nuclear alterations (NA). Additional information was reported in supplementary material.

168 *2.6 Statistical analyses*

169 Univariate analyses were performed with Past v. 4.05 (Hammer et al., 2001) after having
170 checked for data normality and homoscedasticity. Multivariate analyses were performed using
171 PRIMER v. 7 (Clarke et al., 2014) and the PERMANOVA for PRIMER routines (Anderson et al., 2008).

172 2.6.1 Chemical analysis

173 In case of data reported as <LOD, values equal to LOD/2 were attributed according to Cohen
174 and Ryan (1989). Principal component analysis (PCA) tests on normalized data were performed to
175 visualize patterns of contaminant concentrations in crabs' tissues across the four populations.

176 A two-way Permutational Multivariate Analysis of Variance (PERMANOVA: Anderson, 2001)
177 test, based on a matrix built on Euclidean distances and 9999 permutations, was performed to
178 evaluate differences in contaminant content of crab's tissues from populations under different
179 anthropic pressures, i.e., in ports and MPAs (factor status, fixed and orthogonal) across the four study
180 sites (factor site, random and nested in status). In case the factor status was not significant, a one-way
181 PERMANOVA with only the factor site was performed. To ascertain which of the original variables, i.e.,
182 concentrations of contaminants, were mainly responsible for the differences found by the
183 PERMANOVA, we used a Canonical Analysis of Principal Coordinates (CAP, Anderson and Robinson,
184 2003; Anderson and Willis, 2003) as a constrained ordination.

185 2.6.2 Ecological surveys

186 Four χ^2 tests (observed versus expected) were applied to test if the observed sex ratios were
187 discordant from the expected value of 50% in each population. Four χ^2 tests (observed versus
188 observed) were also applied to test differences between the sex ratio values and number of individuals
189 parasitized by *S. carcini* across the populations.

190 A two-way PERMANOVA was applied to test the null hypothesis of no differences in density
191 values between protected and port areas (factor status, port vs MPA, fixed and orthogonal) across the
192 four populations (factor site, random and nested in factor status).

193 Three-way PERMANOVA tests were then applied to test the null hypothesis of no differences
194 in size (expressed as CW, in mm) between sexes (fixed and orthogonal) and ovigerous stage (fixed and
195 orthogonal), respectively, and between protected and port areas (factor status, port vs MPA, fixed
196 and orthogonal) across the four populations (factor site, random and nested in factor status).
197 Regression analyses were applied to test the relationship between the number of embryos at
198 development stage I-II and stage IV-V (log transformed) and the female's size (measured as CW, in
199 mm). A three-way Permutational Analysis of Covariance (PERMANCOVA) was then applied to test the
200 null hypothesis of no differences in number of embryos carried by each female at development stage
201 I-II and IV-V (fixed and orthogonal) between protected and port areas (factor status, fixed and
202 orthogonal) across the four populations (factor site, random and nested in factor status). Females'
203 size (measured as CW, in mm) was used as covariate in the analysis.
204 Since the above-mentioned PERMANOVA and PERMANCOVA analyses, designed with factor
205 site nested in the factor status (port vs MPA), showed a very low influence of the status factor ($P > 0.5$)
206 on the total variation of all the dataset (see supplementary material), this later factor was
207 excluded from the designs to better depict differences among the populations. The analyses were
208 rerun using the factor site as fixed and orthogonal and we report the results of these analyses only.
209 2.6.3 Biomarker analysis
210 To visualize patterns of occurrence of nuclear anomalies across individuals collected from the
211 four study sites, we applied a PCA using similarity matrix based on Euclidean distance on normalized
212 data.
213 Data on nuclear abnormalities (i.e., expressed as frequencies of lobed, apoptotic, kidney,
214 binucleated cells and micronuclei), based on 9999 permutations, were then analyzed applying a three-
215 way PERMANOVA design to evaluate differences between males and females (factor sex, fixed and
216 orthogonal) collected in sites under different anthropic pressures, i.e., in ports and MPAs (factor
217 status, fixed and orthogonal) across the four study sites (factor site, random and nested in status). As
218 the factor status was not significant, we rerun a two-way PERMANOVA test with factors sex and site,

both fixed and orthogonal. This allowed us to better investigate pairwise differentiation between sites, which were then visualized using a CAP based on the same data matrix.

The same statistical approach (three-way PERMANOVA design followed by a two-way PERMANOVA) was also applied to depict differences in enzymatic and physiological biomarkers (AChE, GST, IDH, LDH, CAT, GT, LPO) across male and female crabs collected from the four study sites. Before running each PERMANOVA we tested the homogeneity of multivariate dispersion of data applying a Permutational Analysis of Multivariate Dispersions (PERMDISP: Anderson, 2006: Anderson et al., 2008), and data were properly transformed when necessary.

3. Results

3.1 Chemical analysis

Most of the examined organic contaminants were detected under the LOD value in the crabs' tissue, while heavy metals were more abundant at all sites. The PCA and CAP plots based on both inorganic and organic contaminants show a clear differentiation in concentrations between SM and all the other localities (Fig. 2). Crabs collected from RM and LG were well separated from those of the other two localities and accumulated higher concentrations of PHE and total heavy hydrocarbons (TOTHYDR > 10) (Fig. 2). Considering trace metals bioaccumulation, crabs from SM showed a higher concentration of As, while crabs from PM accumulated higher concentrations of Cd, Cr and Mn (Fig. 1). Crabs collected at the two Ligurian sites, RM and LG, showed high concentrations of Zn, Ni, Pb and Cu (Fig. 1). The one-way PERMANOVA conducted on concentrations of total inorganic and organic contaminants confirmed the strong separation among crabs collected across the four sites (Pseudo $F = 2.97$, $P < 0.001$). Post-hoc pairwise tests showed that PM was different from LG and RM only. As expected by their high abundance in the crabs' tissues, heavy metals were the major drivers of the statistical differences found among sites, with the crabs collected at the Ligurian sites, RM and LG, showing high accumulations for most of the detected trace metals (Fig. 2).

The results of the two-way PERMANOVA including the factor status and the factor site nested in status are reported in Table S1.

245 *3.1 Ecological surveys*

246 3.1.1 Density and population structure

247 The number of captured crabs for each population is reported in Table 1. Despite at all sites,
248 except RM, males outnumbered females (Table 1), the sex ratio values never differed significantly
249 from a 50% value (χ^2 tests: PM, $\chi^2 = 2.94$, df = 1, P= 0.09; SM, $\chi^2 = 1.74$, df = 1, P = 0.17; LG, $\chi^2 = 3.49$,
250 df = 1, P = 0.06; RM, $\chi^2 = 0.23$, df = 1, P = 0.63). The sex ratio values (Table 1) did not differ among the
251 four populations as well (χ^2 test: $\chi^2 = 6.71$, df = 3, P = 0.08).

252 Berried females were more abundant in RM and LG than in SM and PM (Table 1). The numbers
253 of berried females were significantly different among the four populations (χ^2 test: $\chi^2 = 14.18$, df = 3,
254 P = 0.002).

255 The proportion of crabs parasitised by *S. carcini* ranged from less than 1% (in SM) to about
256 25% (in LG) (Table 1), with the number of parasitised individuals strongly differing among the four
257 populations (χ^2 test: $\chi^2 = 42.53$, df = 3, P < 0.001).

258 The crab density was much higher in PM than in the other three populations (Table 1). The
259 one-way PERMANOVA recorded a significant difference in densities across the four populations
260 (Pseudo-F = 57.69, df = 3, P < 0.0001). Post-hoc pairwise tests showed that all comparisons were highly
261 significant (P = 0.0001) except from SM versus RM (P = 0.82).

262 Range-size distribution patterns differed across the four populations, both in terms of average
263 crab size and size ranges (Fig. 1). The two-way PERMANOVA performed on the log transformed CW
264 data showed a significant influence of the factor site, but not for the factor sex (Table 2). The
265 interaction between the two factors was, however, significant and the pairwise tests indicated that
266 females' average CW and males' average CW differed between all the population pairs except for SM
267 versus LG and SM versus PM, respectively.

268 Range-size distributions of ovigerous and non-ovigerous females differed across the four
269 populations, as showed by the two-way PERMANOVA test (Table 2). As evident from Figure 1, SM is

the only population where only females larger than 20 mm were ovigerous, while in the other sites berried females belonged to all size classes.

3.1.2 Fertility and fecundity estimation

The number of embryos carried by a female ranged from a minimum of 1,646 to a maximum of 107,684, the amount found on a female with a CW of 35.9 mm collected in LG. Medium-size females (CW around 20-25 mm) carried on average 27,500 ± 1,861.05 embryos. The average reproductive output for each population, expressed as Ratio_Fe and Ratio-PoFe per CW ratio, are reported in Table S2.

Regardless of the degree of development of the embryos, we confirmed the positive relationship between crab's female dimensions and egg production (Regression test on cumulated data: $F = 225.43$, $df = 33$, $P < 0.0001$ and $F = 109.48$, $df = 30$, $P < 0.0001$ for I-II stage and IV-V stage, respectively. Fig. S1).

The two-way PERMANCOVA test indicated that there is a significant difference in the number of fertilized eggs produced by the females collected in the four sites (Table 3). The Pairwise tests indicated that the embryos production differed only between SM and LG ($t = 3.10$, $P = 0.005$). Conversely, the developmental stage of the embryos did not have any significant effect on the number of embryos carried by a female (Table 3). The covariate CW, as expected, significantly affected the number of embryos carried.

The results of the PERMANOVAs and PERMANCOVA including the factor status (see section 2.6.2) are reported in supplementary material, Tables S3-S6.

3.2 Biomarker analysis

A battery of biomarkers (AchE, IDH, LDH, LPO; GST, CAT, GSH, NA assay) was measured to assess different endpoints in the 148 collected samples. Average values ± standard errors for each biomarker are reported in Table S7. Since any three-way PERMANOVA applied to these data did not show a significant effect of the factor status, hereafter we presented only the results of the two-way

295 PERMANOVA design. Three-way PERMANOVA results are reported in Table S8 of the supplementary
296 material.

297 3.2.1 Genotoxicity

298 Crabs collected at SM showed the lowest mean value of total abnormalities (NA), while the
299 crabs collected in RM the highest one (Table S7, Fig. 3a).

300 The two-way PERMANOVA performed on the frequencies of nuclear abnormalities (lobed,
301 apoptotic, kidney, binucleated cells and micronuclei) recorded a significant differentiation among
302 crabs collected across the four sites, independently from their sex (Table 4). The pairwise tests
303 indicated that all the comparisons between sites were significant except RM and LG. This is confirmed
304 by the results of PCA and CAP that clearly separated the crabs collected from each site except RM and
305 LG (Fig. 4). The ordinations show that the separation is due to a high frequency of lobate cells in the
306 port PM and a prevalence of apoptotic cells in crabs from the port LG and the MPA RM.

307 3.2.2 Neurotransmission and energetic metabolism

308 Acetylcholinesterase activity (AChE) showed the highest average inhibition values in
309 specimens from the site RM (Table S7; Fig. 3b). The two-way PERMANOVA recorded a significant effect
310 of both factors and of their interaction (Table 4). Males from RM had significant lower AChE values (av
311 ± SE = 4.88 ± 0.57 umol/min/mg prot) in comparison to the males collected at the other sites.
312 Moreover, the mean value of activity recorded for females from PM (13.65 ± 1.70 µmol/g tissue/ min)
313 was significantly higher than those recorded for females sampled at the other sites (SM females = 4.43
314 ± 1.53; LG females = 10.43 ± 1.49; RM females = 6.51 ± 1.33).

315 No significant differences were found between crabs collected from different sites when LDH
316 values were considered, while the two-way PERMANOVA showed that females have higher mean
317 values with respect to males (8.77 ± 1.56 and 4.31 ± 0.71, respectively) (Table 4; Fig. 3c).

318 The IDH values differed among crabs collected from different sites but not between sexes.
319 Crabs from PM port showed the highest mean value (56.82 ± 10.93 nmol/min/mg prot) and this value

was statistically different from those recorded for crabs from RM and LG ($p = 0.05$ and $p = 0.002$, respectively) (Table 4; Fig. 3d).

3.2.3 Biotransformation, antioxidant defences and oxidative damage

Regarding LPO and GST levels, no statistical differences among populations and between sexes were recorded by the two-way PERMANOVAs (Table 4; Fig. 3e-f). The GSH levels, instead, were affected by the sampling site (Table 4). The mean values of GSH measured in crabs from RM (1.28 ± 0.44 nmol/min/mg prot) and LG (1.07 ± 0.37 nmol/min/mg prot) were about nine and three times lower with respect to those measured in crabs from PM (9.41 ± 1.29 nmol/min/mg prot) and SM (3.71 ± 1.07 nmol/min/mg prot), respectively. Post-hoc pairwise tests showed a significant difference between each pair of sites, except from RM and LG (Fig. 3g).

The two-way PERMANOVA showed a significant effect of the factor site and of the interaction factor sex × site on values of catalase (Table 4). The pairwise tests indicated that males from PM port significantly differed from males sampled at the other sites; while females' CAT values at the MPA SM were significantly different from the crabs collected at the other sites (Fig. 3h).

4. Discussion

This study emphasizes that *P. marmoratus* is an excellent bioindicator for monitoring and evaluating the environmental quality of natural rocky shores and artificial coastal areas, since the populations sampled at the polluted sites steadily showed the highest signals of stress responses at various biological levels. First, we confirmed that this species accumulates organic and inorganic contaminants in its tissues, as already reported by various authors (Baratti et al., 2022; Fratini et al., 2008; Mouneyrac et al., 2001; Shaeiek et al., 2018). We also validated its resistance to various sources of environmental pollution since we found dense and viable populations at both unpolluted and polluted sites. We also proved that the populations of *P. marmoratus* characterized by the highest levels of heavy metal bioaccumulation showed high levels of vulnerability to parasites, which we interpret as a signal of impairment of the standard development and reproduction cycle, coupled with cellular and enzymatic responses to stressful and energy-demanding conditions. These chemical and

346 biological parameters can indeed be used as an integrated array of ecological and biochemical
347 biomarkers of environmental quality. We finally proved that one of the MPA selected for this study
348 (Riomaggiore located within the Cinque Terre National Park) shows the same pressure from
349 contaminants than the adjacent port site (Le Grazie within the La Spezia harbor) since crabs collected
350 at these two sites shared similar contaminant levels and biomarker responses.

351 Our analyses revealed that all sampled crabs, independently from the protection status of the
352 collection sites, showed some evidence of bioaccumulation of heavy metals, while organic compounds
353 were often detected in traces. The levels of bioaccumulation we detected reveal that the two port
354 areas and one of the protected sites, Riomaggiore, show persistent signals of contamination. We also
355 recorded a different chemical signature across sites. Crabs collected from Porto Mediceo of Livorno
356 accumulated higher concentrations of Hg, Cd, Cr, V, Mn, ANA and PYR; while the northernmost sites
357 of Le Grazie and Riomaggiore showed the highest values of Zn, Ni, Pb, Cu, Fe, PHE and total heavy
358 hydrocarbons, irrespective of the protection status of one of those sites. All these trace metals (Cu,
359 Fe, Ni, Pb, Zn, Mn and Hg, in particular) are well known indicators of port contamination and, when
360 accumulated in animal tissues, become toxic and induce the generation of reactive oxygen species
361 and apoptotic processes (Ercal et al., 2001). In a recent study on the transcriptomic patterns of *P.*
362 *marmoratus* shown by individuals collected at two of our study sites (Porto Mediceo of Livorno and
363 Secche della Meloria), Baratti et al. (2022) recorded an up-regulation of metabolic pathways
364 implicated in the defense against reactive oxygen species in individuals from the port area, due to
365 contaminant exposure. Our results also showed that crabs from the MPA Secche della Meloria were
366 characterized by the lowest levels of bioaccumulation of the studied contaminants, except for arsenic.
367 This finding can be explained by the peaks of arsenic in the Livorno port area recorded by Leoni and
368 Sartori (1996), who also noticed that the sediments of the MPA Secche della Meloria were enriched
369 in As, V, Cu and Pb. This also confirms what already reported by Fratini et al. (2008), who recorded
370 similarly high levels of bioaccumulation of As in *P. marmoratus* crabs from polluted and unpolluted

371 sites across the Tuscany coast, underlying that “virtually no areas along the coast of this Italian region
372 are safe from heavy metal pollution”.

373 Our ecological surveys showed some differences in terms of density, population structure and
374 size, and reproductive investment among the four populations, which can be related to differences in
375 environmental factors, such as food and refuges availability, as well as in microhabitat structure. Some
376 of these differences, however, can be imputable to exposure to contaminants, in line with published
377 studies that show how crab morphometrics, population dynamics, and behaviors may be indicators of
378 environmental health (Bergey and Weis, 2008; Cannicci et al., 2009; Giblock and Crain, 2013;
379 Mansfield, 2009; Wildsmith et al., 2009). The population of Porto Mediceo of Livorno is the densest;
380 while the population of Le Grazie hosts the biggest individuals, being the lowest in density. Both a high
381 density and a large size can be explained by the large availability of food linked to the presence of
382 organic contaminants causing overabundance of algae, the main food source for *P. marmoratus*
383 (Cannicci et al., 2002, 2007). Two very interesting parameters differentiated the population of Secche
384 della Meloria, the least impacted site in terms of contaminants., from the other ones. First, this
385 population is the only one with a very low incidence (less than 1%) of the parasite *S. carcini*, with the
386 other populations showing incidences between 10% and 30%. *S. carcini* castrates both male and
387 female crabs and feminizes the males affecting the reproductive viability of the parasitized
388 populations (Thresher et al., 2000). A higher incidence of parasites may be due to a higher
389 susceptibility of the host to infections that can be due to the level of chemical pollution, which impacts
390 their immuno-competence (Labaude et al., 2015; Lafferty and Kuris, 1999). Secondly, in the Secche
391 della Meloria population, the ovigerous females are always medium- to big-sized crabs (CW > 20 mm),
392 while in the other three populations we also found very small ovigerous females (from CW ≥ 10 mm).
393 We hypothesize that small females living in a high-quality environment, such as Secche della Meloria,
394 experience low levels of metabolic stress and invest energy in growth, to start reproducing only when
395 they attain a larger size. This strategy is an evolutionary stable condition, since the number of eggs
396 produced by a female exponentially increases with the linear increase of its size (Jouili et al., 2016;

397 Öndes et al., 2002). In this study we also investigated if anthropogenic impacts could induce a
398 reduction in production of embryos and their survival, as recorded for other intertidal crabs (see
399 Penha-Lopes et al., 2009). This effect is not evident for *P. marmoratus* whose females do not lose eggs
400 during embryonic development when living in human impacted sites, i.e., in stressful, energy-
401 demanding conditions. Anyway, we cannot exclude that human impact could affect the maternal
402 nutritional investment during oogenesis, and consequently the quality of hatched larvae and their
403 survival.

404 In accordance with the ecological surveys, the results of the biomarkers indicated the
405 occurrence of cellular and enzymatic responses to stressful and energy-demanding conditions in *P.*
406 *marmoratus* individuals that showed the highest bioaccumulation levels of heavy metals. The
407 biomarker responses we recorded were generally within the range previously reported in field studies
408 on crabs (Duarte et al, 2017a; Elumalai et al., 2007; Locatello et al., 2009; Ragunathan, 2017; Ricciardi
409 et al., 2010; Rodrigues et al., 2012, 2014; Tsangaris et al., 2016), demonstrating a good sensitivity of
410 the various tests. This is the first study that investigated micronuclei and other cellular abnormalities
411 in *P. marmoratus*. The low average values of NA assay found at the MPA Secche della Meloria are in
412 line with the relatively low values of metals and PAHs found in this MPA, demonstrating the limited
413 impact of contaminants that are usually responsible for the genotoxic effects on organisms. The
414 highest frequency of micronuclei, which is considered an irreversible damage, was found in crabs
415 sampled in the Porto Mediceo of Livorno and these data are supported by high concentrations of Hg,
416 Cd, Cr, V, Mn, ANA and pyrene. Since pyrene is one of the most abundant PAHs in the environment
417 (Nudi et al., 2010; Stroomberg et al., 1999) and Hg, Cd and Cr are among the most genotoxic metals
418 (García-Medina et al., 2017), the results of the NA assay are evidence of a toxicological risk related to
419 the presence of these compounds in this area.

420 The evaluation of the AChE activity inhibition is widely used as a biomarker of exposure to
421 neurotoxic compounds (such as organophosphates and carbamates) in invertebrates, including crabs
422 (Ricciardi et al., 2010). Other classes of contaminants can alter AChE synthesis pathway by reducing

its production. Previous studies showed that AChE could be inhibited by metals (Elumalai et al., 2002; 2007; Rodrigues et al., 2012) such as Cu, Zn e Cd (Frasco et al., 2005); on the other hand, some PAHs are able to induce metabolic processes that bioactivate some organophosphorus compounds (Belden and Lydy, 2000; Binelli et al., 2006). We recorded the highest values of AChE inhibition in individuals collected at Riomaggiore. At this site, the presence of pesticides coming from agricultural sources could be considered, as Liguria is a region where large cultivations of flowers and fruits are present. The highest levels of Cu and total PAHs, combined with the highest level of AChE inhibition, could also be attributed to organic contaminants released by the very large number of boats transporting hundreds of tourists every day to Riomaggiore.

The lactate dehydrogenase (LDH) enzyme plays an important role in several metabolic pathways. In anaerobic glycolysis, LDH is the terminating enzyme in the sequence of reactions promoting the breakdown of glucose into lactate and it is essential for ATP production. It becomes particularly important when a significant amount of additional energy is promptly required. The higher values found in females compared to males are explained by the well-known higher energetic investment in oogenesis and brood care performed by females during the reproductive period. LDH is also used in ecotoxicology as a general indicator of tissue and cellular damage (Diamantino et al., 2001). Although the use of this enzyme as a biomarker in aquatic invertebrates has been poorly investigated (Menezes et al., 2006), environmental contaminants such as metals, PAHs, and pesticides are known to cause an increased LDH activity (Guimarães et al., 2012; Vijayavel and Balasubramanian, 2006). The LDH values recorded in this work are lower than those obtained in other field studies on crabs (Luis and Guilhermino, 2012; Rodrigues et al., 2012, 2014). The low values we obtained and the lack of difference between our sampling sites may indicate that the animals are not in hypoxic conditions, as this enzyme operates only under aerobic conditions. The isocitrate dehydrogenase (IDH) is an enzyme that plays a key role in cellular defense against oxidative damage produced by reactive oxygen species (ROS). An induction of IDH activity could be due to a general increase of the citric acid cycle function in response to a demand of energy for detoxification at high concentrations of

449 contaminants or it might be a response to an increased oxidative stress requiring additional
450 regeneration of NADPH (Rodrigues et al., 2012). Highest values of IDH measured in the crabs collected
451 in Porto Mediceo of Livorno, where also the total glutathione showed the highest values, suggest that
452 only this population is subjected to an evident oxidative stress.

453 Aquatic organisms, including crabs, developed an antioxidant defense mechanism to mitigate
454 oxidative stress caused by a wide variety of environmental and anthropogenic stressors. This
455 antioxidant defense system, that include a wide battery of enzymatic (superoxide dismutase, SOD;
456 catalase, CAT; glutathione peroxidase, GPX; glutathione reductase, GR; glutathione-S-transferase,
457 GST) and non-enzymatic antioxidants (total glutathione, GSH and ascorbic acid), is one of the
458 biochemical mechanisms that protect the organism from cellular damage caused by ROS through a
459 cascade of biochemical reactions (Ragunathan, 2017). The hemoprotein CAT increases the removal of
460 H_2O_2 generated during the reaction of oxyradicals with SOD. Again, the highest CAT values were
461 measured in crabs sampled in Porto Mediceo of Livorno and could indicate the presence of high levels
462 of ROS. In addition, higher GSH levels were measured at the same site, presumably due to increased
463 free radical activated oxidative damages from exposure to contaminants. On the other hand, it could
464 be an adaptive mechanism of *P. marmoratus* to mitigate oxidative stress. Reduction of peroxides using
465 GSH leads to protection from oxidation of membrane lipids (Aniagu et al., 2006). The absence of an
466 increase in the levels of LPO in the samples coming from Porto Mediceo of Livorno, as from the other
467 sites, could confirm this hypothesis. Overall, these results suggest that specimens from this port site
468 would generally be under higher oxidative stress, and this could be caused by high levels of
469 contaminants such as Hg, Cd and PAHs.

470 **5. Conclusion**

471 To our knowledge, this is the first attempt to apply an integrated multidisciplinary approach
472 based on ecological surveys, chemical analyses, cellular and multi-biomarkers responses on an
473 intertidal crab species with the aim to investigate the possible effects of the environmental
474 contamination on marine sites subjected to different anthropogenic impacts. The obtained results

proved that our approach is a valuable tool in the health assessment of protected and disturbed Mediterranean coastal environments. Crabs collected in the MPA Secche della Meloria did not show any stress-related response, while the crabs from the adjacent port area of Livorno showed the highest levels of genotoxic and oxidative stress effects among the tested ones. On the other hand, crabs collected at the two Ligurian sites did not differ from each other in terms of heavy metal bioaccumulation and biomarkers responses, even if they were less impacted than those collected in the industrial port of Livorno. This suggests that Riomaggiore, although included in an MPA, is impacted by contaminants from adjacent polluted areas, confirming that protected areas are unable to prevent the spread of pollutants by means of superficial currents. Our results show that the bioaccumulation capabilities of a ubiquitous sentinel species and its biological responses to pollutants can uncover subtle but significant impacts on protected populations and are essential tools to inform efficient management and protection schemes for coastal habitats.

Author contribution statement

Caliani Ilaria, Cannicci Stefano and Fratini Sara: Conceptualization, Methodology, Data Curation, Supervision, Writing- Original draft preparation; Pretti Carlo, Baratti Mariella, Contini Ginevra, Vitale Matteo, Iannucci Alessio: Methodology, Investigation, Writing- Reviewing and Editing; Silvia Casini and Maria Cristina Fossi: Writing Reviewing and Editing

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Figure captions:

Figure 1. A) Map of the study sites (modified from Iannucci et al., 2020). Surveys on *Pachygrapsus marmoratus* populations and collection of individuals for population structure, biochemical and toxicological analyses were made in four sites of the Ligurian Sea: Porto Mediceo (PM), Secche della Meloria (SM), Riomaggiore (RM), Le Grazie (LG). B) Range-size frequency distributions of *Pachygrapsus marmoratus* males and females as well as of berried and not- berried females recorded at the four study sites. Size is expressed as carapace width, CW, in mm.

Figure 2. Principal component analysis (PCA) and Canonical Analysis of Principal Coordinates (CAP) plots based on the concentrations of total inorganic and organic contaminants found in the tissues of *Pachygrapsus marmoratus* individuals collected in the four sampling sites. Vectors of the linear correlations between individual variables are superimposed on the graphs. Site abbreviations as in Figure 1.

Figure 3. Boxplots of the different biomarkers recorded in *Pachygrapsus marmoratus* individuals collected in the four sampling sites. Site abbreviations as in Figure 1.

Figure 4. Principal component analysis (PCA) and Canonical Analysis of Principal Coordinates (CAP) plots showing the distribution of *Pachygrapsus marmoratus* samples from the four study sites in relation to nuclear and cellular abnormalities. Vectors of the linear correlations between individual variables are superimposed on the graphs. Site abbreviations as Figure 1.

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Parameters	SM (43.546 N, 10.218 E)	PM (43.549 N, 10.294 E)	RM (44.096 N, 9.739 E)	LG (44.067 N, 9.841 E)
Total crabs	138	201	140	122
Males	72	110	66	71
Females	50	86	74	43
Berried females (%)	12 (24.0)	14 (16.28)	31 (41.89)	15 (34.88)
Juveniles	16	5	0	8
Sex ratio %	59.02	56.12	47.14	62.28
Ind. sacculinised (%)	1 (0.72)	20 (9.95)	10 (7.14)	30 (24.59)
Density (ind/m^2)	3.09 ± 0.48	10.29 ± 0.71	3.27 ± 0.53	0.38 ± 0.16

771

772 **Table 1**. Number of *Pachygrapsus marmoratus* individuals captured at the four study sites, Secche
773 della Meloria (SM), Porto Mediceo (PM), Riomaggiore (RM) and Le Grazie (LG). Density and sex ratio
774 (calculated as n. of male/total number of individuals * 100) were also reported. Juveniles indicated
775 small individuals whose sex could not be determined. The geographic coordinates are reported for
776 each site.

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	Source	df	SS	MS	Pseudo-F	P(perm)
SEX	Site (Si)	3	7.99	2.67	44.29	**< 0.001**
	Sex	1	0.005	0.005	0.09	0.77
	Si x Sex	3	0.69	0.23	3.80	**0.01**
	Residual	564	33.95	0.06		
	Total	571	43.52			
OVIGEROUS STAGE	Site (Si)	3	3.07	1.02	22.62	**< 0.001**
	Ovigerous stage (Ov)	1	0.12	0.12	2.60	0.11
	Si x Ov	3	0.34	0.11	2.54	0.06
	Residual	245	11.10	0.04		
	Total	252	14.33			

Table 2. Results of the two-way PERMANOVA conducted on crabs' sizes (expressed as carapace width, CW, in mm) of male and female individuals, and of berried and no-berried females across the four populations. The following abbreviations are used: df, degree of freedom, SS, sum of squares, MS, mean squares, P(perm), probability values calculated through 9999 permutations. Significant P values are in bold.

Source	df	SS	MS	Pseudo-F	P(perm)
CW (covariate)	1	33.48	33.48	308.81	**< 0.001**
Site (Si)	3	0.96	0.32	2.94	**0.036**
Development stage (Dev)	1	0.01	0.007	0.06	0.80
Si x Dev	3	0.24	0.08	0.73	0.54
Residual	56	6.07	0.11		
Total	64	40.75			

Table 3. Results of the two-way PERMANCOVA conducted on the number of embryos at the first and last stages of development carried by each female (data log transformed) across the four populations. The size of the female (expressed as carapace width, CW, in mm) is used as covariate. The following abbreviations are used: df, degree of freedom, SS, sum of squares, MS, mean squares, P(perm), probability values calculated through 9999 permutations. Significant P values are in bold.

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	Source	df	SS	MS	Pseudo-F	P(perm)
NA	Sex	1	335.47	335.47	10.80	0.116
	Site (Si)	3	4837.20	1612.40	2.25	**< 0.001**
	Sex X Si	3	386.51	128.84	0.86	0.496
	Residual	61	9107.50	149.30		
	Total	68	14618			
AChE	Sex	1	156.04	156.04	5.23	**0.026**
	Site (Si)	3	698.19	232.73	7.80	**< 0.001**
	Sex X Si	3	296.85	98.95	3.32	**0.026**
	Residual	70	2089	29.84		
	Total	77	3212.4			
LDH	Sex	1	5.57	5.57	9.67	**0.003**
	Site (Si)	3	0.54	0.18	0.31	0.819
	Sex X Si	3	2.91	0.97	1.69	0.178
	Residual	63	36.25	0.58		
	Total	70	44.29			
IDH	Sex	1	363.64	363.64	0.56	0.453
	Site (Si)	3	8870.30	2956.80	4.55	**0.005**
	Sex X Si	3	599.17	199.72	0.31	0.822
	Residual	63	40910	649.36		
	Total	70	51181			
GST	Sex	1	45.121	45.12	0.02	0.892
	Site (Si)	3	3950.1	1316.70	0.52	0.667
	Sex X Si	3	2948.2	982.75	0.39	0.760
	Residual	86	2.2E+05	2526.90		
	Total	93	2.2E+05			
LPO	Sex	1	5.96	5.96	0.87	0.354
	Site (Si)	3	37.72	12.57	1.84	0.146
	Sex X Si	3	44.97	14.99	2.19	0.094
	Residual	93	656	6.83		
	Total	103	740.03			

GSH	Sex	1	1.90	1.90	0.25	0.631
	Site (Si)	3	515.33	171.78	22.17	**< 0.001**
	Sex X Si	3	28.78	9.59	1.24	0.306
	Residual	46	356.48	7.75		
	Total	53	954.19			
CAT	Sex	1	0.01	0.01	0.02	0.891
	Site (Si)	3	5.44	1.81	4.30	**0.008**
	Sex X Si	3	6.24	2.08	4.93	**0.004**
	Residual	77	32.52	0.42		
	Total	84	45.81			

Table 4. Results of the two-way PERMANOVAs conducted on conducted on frequencies of lobed, apoptotic, kidney, binucleated cells and micronuclei, total cellular abnormalities (NA) and on physiological and enzymatic biomarkers recorded for male and female crabs collected across the four study sites. The following abbreviations are used: df, degree of freedom, SS, sum of squares, MS, mean squares, P(MC), probability values calculated through 9999 permutations and the Monte Carlo test. Significant P values are in bold.

813 A)

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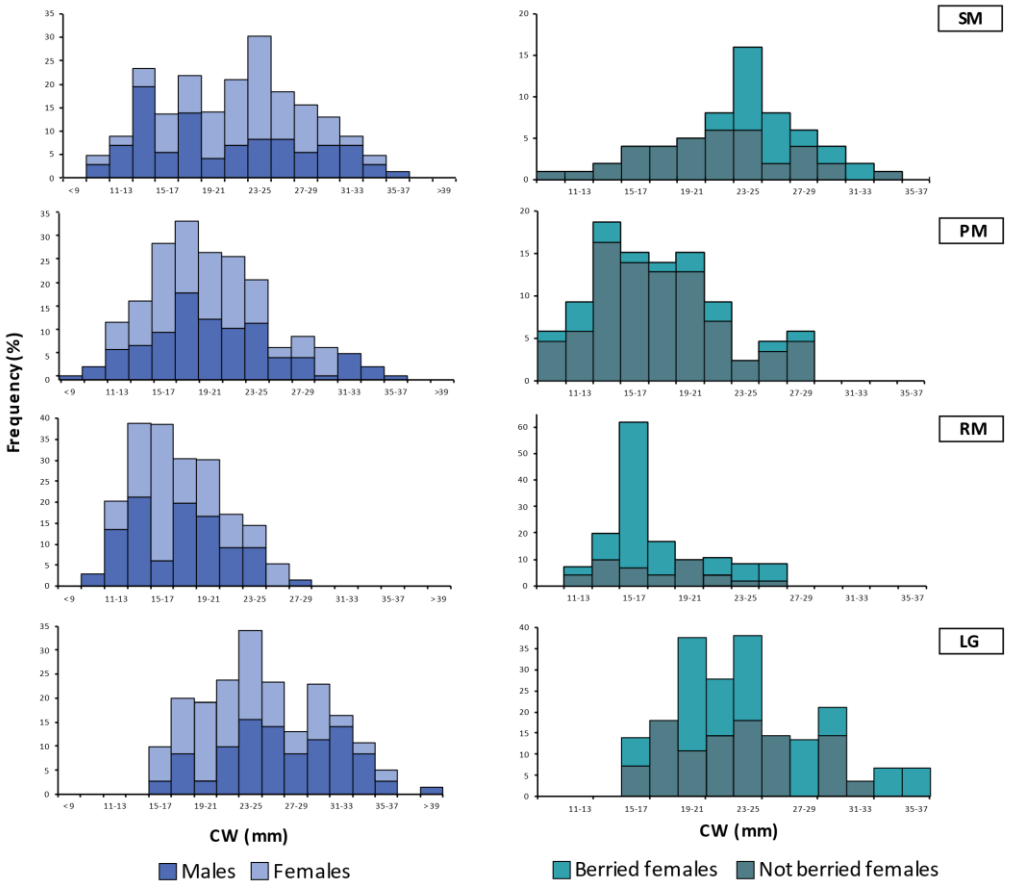
815 B)



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817 **Figure 1**

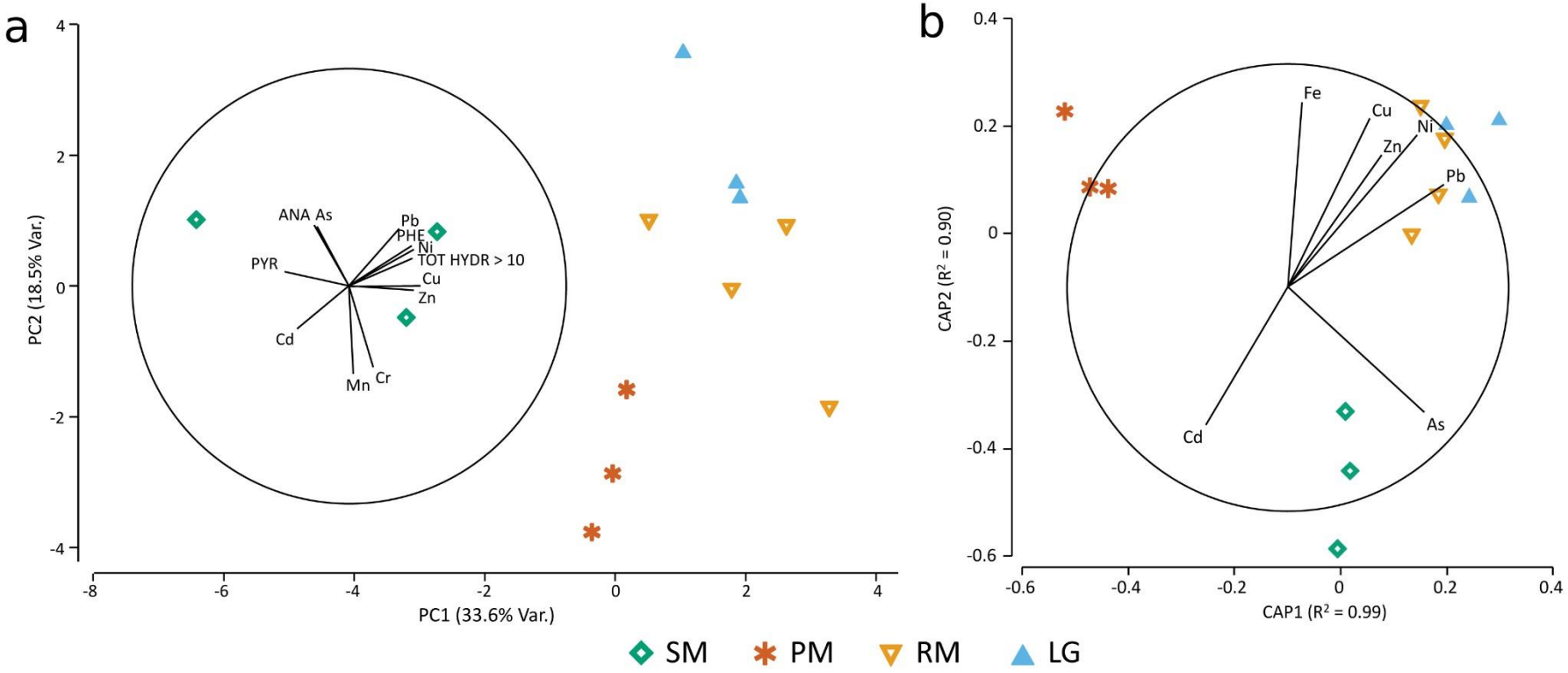
a
b
PC2 (18.5% Var.)
PC1 (33.6% Var.)
ANA As
Pb
PHE
Ni
TOT HYDR > 10
PYR
Cu
Zn
Cd
Mn
Cr
CAP2 (R² = 0.90)
CAP1 (R² = 0.99)
Fe
Cu
Ni
Zn
Pb
Cd
As
SM
PM
RM
LG

Figure 2

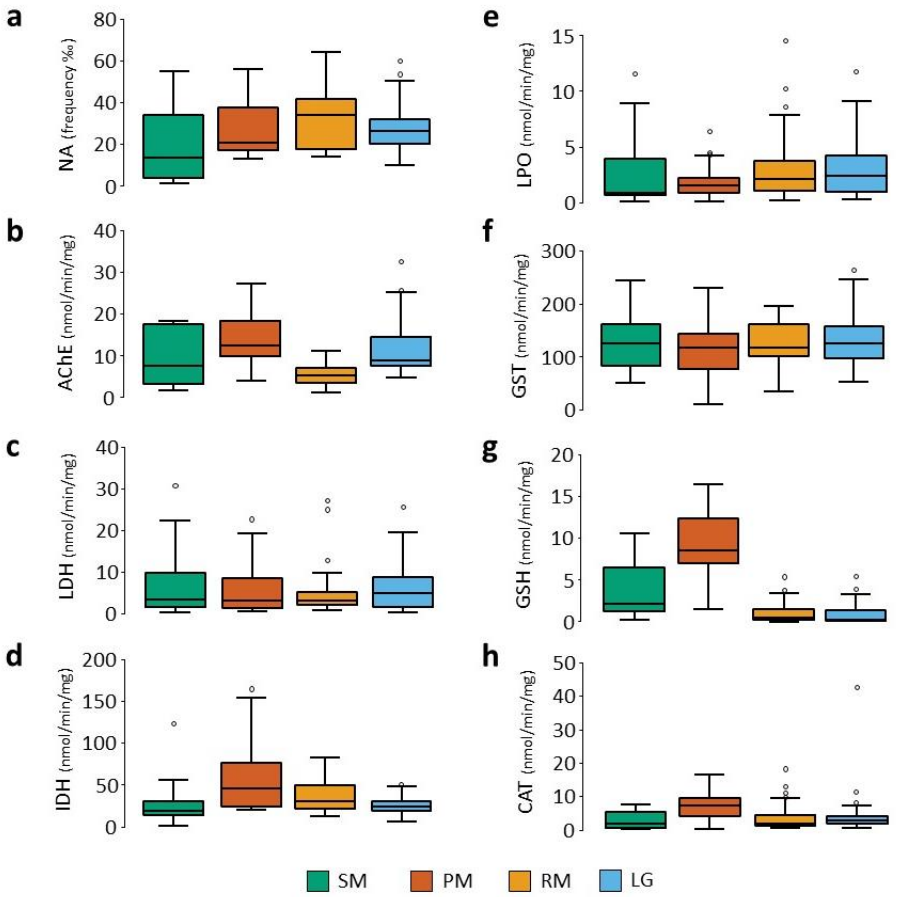
a
NA (frequency ‰)
b
AChE (nmol/min/mg)
c
LDH (nmol/min/mg)
d
IDH (nmol/min/mg)
e
LPO (nmol/min/mg)
f
GST (nmol/min/mg)
g
GSH (nmol/min/mg)
h
CAT (nmol/min/mg)
SM
PM
RM
LG

Figure 3

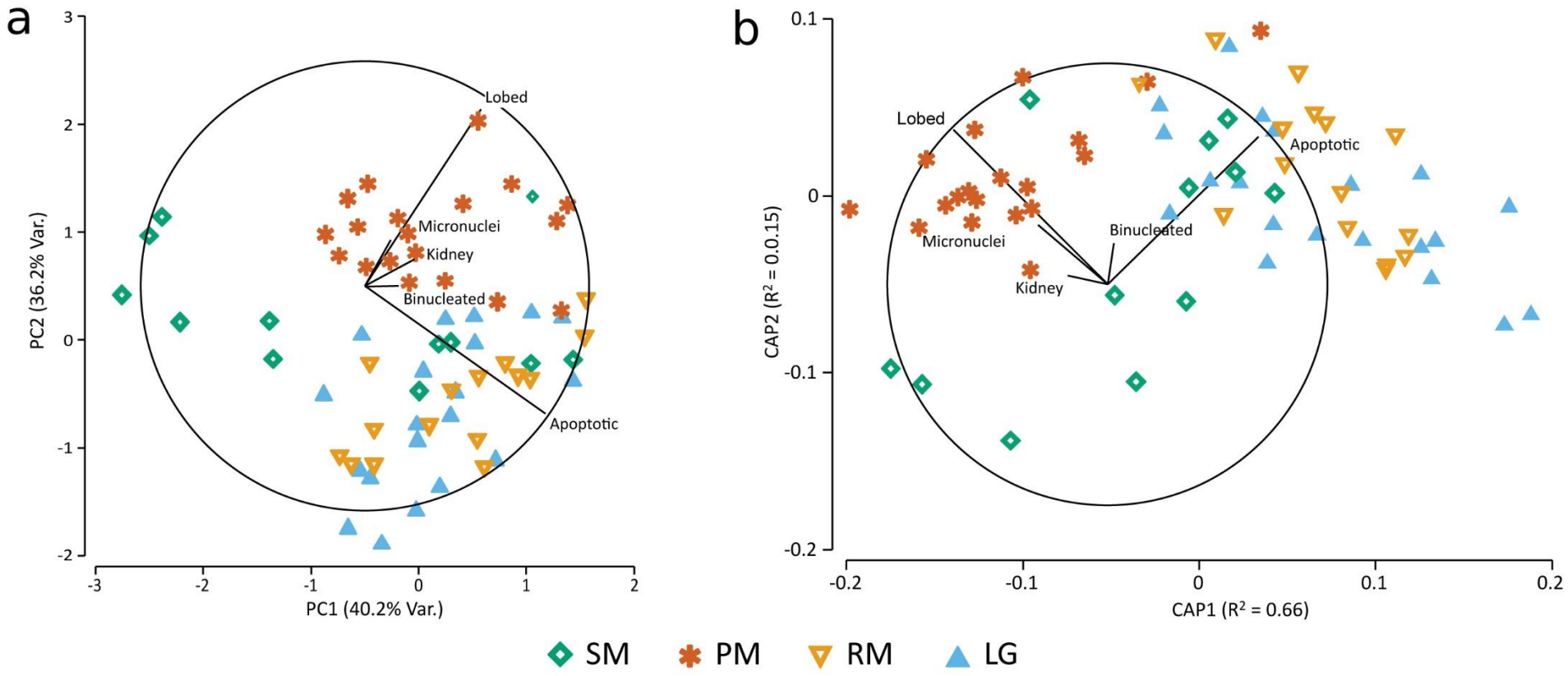
a
b
Lobed
Micronuclei
Kidney
Binucleated
Apoptotic
PC1 (40.2% Var.)
PC2 (36.2% Var.)
CAP1 (R² = 0.66)
CAP2 (R² = 0.0.15)
SM
PM
RM
LG

Figure 4

Highlights

- Ecological, chemical and biomarker data were recorded in an intertidal crab
- Populations from two marine protected areas and two adjacent ports were selected
- Oxidative stress and genotoxicity biomarkers were higher in crabs from polluted sites
- One protected population showed stress related responses similar to adjacent port one
- Our integrated approach showed to be reliable for efficient management for coastal habitats

Author contribution statement

Caliani Ilaria, Cannicci Stefano and Fratini Sara: Conceptualization, Methodology, Data Curation, Supervision, Writing- Original draft preparation; Pretti Carlo, Baratti Mariella, Contini Ginevra, Vitale Matteo, Iannucci Alessio: Methodology, Investigation, Writing- Reviewing and Editing; Silvia Casini and Maria Cristina Fossi: Writing Reviewing and Editing

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Graphical Abstract