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1 **First characterisation of seasonal variations in biomarkers baseline in *Patella* sp. from**  
2 **Mediterranean ports (North Corsica, France)**

3

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17

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21

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27

28 **Abstract**

29 In port areas the identification of contamination sources is necessary for an efficient  
30 management. Biomonitoring provides information on the environmental impact of the  
31 pollutants. It is often difficult to differentiate the natural variations of biomarkers from those  
32 induced by pollution. The present study aims to define a baseline level for biochemical  
33 biomarkers in limpet (*Patella* sp.) collected in four North-Corsica port areas. Reference data  
34 for five biomarkers (superoxide dismutase, glutathione S-transferase, laccase, pyruvate kinase  
35 and acetylcholinesterase) were described in a model, using length of the limpet shell,  
36 temperature and salinity. The measured biomarkers responses on potentially polluted sites  
37 usually fell within the range of the expected values for an unaffected site, suggesting that a main  
38 part of the variations is explained by environmental conditions. Not included in the model,  
39 biological factors (sex, development stage, etc.), annual variation or other physico-chemical  
40 parameter could explain the variations in the model.

41

42

43 Keywords: biomonitoring, baseline assessment criteria, biochemical biomarkers, *Patella* sp.

44

45

46 As a semi-enclosed sea affected by a growing coastal urbanization, the Mediterranean Sea is  
47 the recipient of numerous anthropic pollutants (trace elements, organic and emergent  
48 compounds). In addition to the pollutants coming from terrestrial discharges or anthropogenic  
49 contamination (Benali et al., 2015; Lafabrie et al., 2008), port areas are also impacted by  
50 recreational activities, adding further pressure on the ecosystem (Maisano et al., 2017). To set  
51 up an effective environmental management of port areas, it is important to define the sources  
52 of the pollution and its impacts on the ecosystem. This is why the monitoring of coastal  
53 environment is essential for policy makers involved in the European Water Framework  
54 Directive (WFD). However, monitoring natural marine ecosystems regarding chemical  
55 mixtures is challenging and remains until today one of the major challenges in ecotoxicology.  
56 Biomonitoring consists on the study of geographical and temporal trends of contaminants  
57 through biological responses of an organism (Lacroix et al., 2015; Rainbow, 2002). It has been  
58 developed to identify sources of pollution and prevent risks of future potential pollution  
59 accidents. Compared to simple chemical water analysis, biomonitoring is a time-integrated  
60 method and allows the evaluation of effects of contaminants on organism health (Lacroix et al.,  
61 2015; Luna-Acosta et al., 2015; Rainbow, 2002; Wang, 2016). Limpets (*Patella* sp.) are more  
62 and more used for biomonitoring studies and is considered as a promising bioindicator (Pérez  
63 et al., 2019; Reguera et al., 2018) due the its abundance, its sedentary lifestyle, its large  
64 distribution or its ease of sampling (Campanella et al., 2001; Pérez et al., 2019). Moreover, the  
65 use of native biota as a monitoring tool for bioavailable pollutants in aquatic ecosystems was  
66 demonstrated to constitute a highly valuable approach (Bertrand et al., 2018). Limpets are now  
67 widely used in biomonitoring studies (Pérez et al., 2019; Reguera et al., 2018; Sánchez-Marín  
68 et al., 2022; Viñas et al., 2018) and their high sensitivity to pollutants was demonstrated (Conti  
69 et al., 2017; Reguera et al., 2018).

70 In environmental study, when a mixture of contaminants is present, it is recommended to use a  
71 multi-biomarkers approach and to consider the environmental physicochemical parameters, to  
72 differentiate the reversible effects from the irreversible ones (Garmendia et al., 2011). This  
73 approach provides a better understanding and a more integrated view of the biological responses  
74 in environmental risk assessment (Cravo et al., 2009; Ozkan et al., 2017). The efficiency of the  
75 multi-biomarkers approach has been proven by several authors (Ait Alla et al., 2006; Damiens  
76 et al., 2004; Garmendia et al., 2011; Guo et al., 2017; Vidal et al., 2002).

77 A wide diversity of biomarkers is used in a multi-biomarkers approach but the most common  
78 are usually enzymes involved in antioxidative defences (Breitwieser et al., 2018; Ghribi et al.,  
79 2020; Luna-Acosta et al., 2017b), in nervous system (Lionetto et al., 2013; Lionetto et al., 2003;  
80 Matozzo et al., 2005), in immunity (Luna-Acosta et al., 2017a) or in energetic metabolism  
81 (Falfushynska et al., 2019; Zebral et al., 2020). However, it is also demonstrated that biotic  
82 factors (length, weight, reproductive cycle, etc.) (Louis et al., 2021), and abiotic factors  
83 (temperature, salinity, etc.) (Castaldo et al., 2021; Prusina et al., 2014; Torossian et al., 2020;  
84 Uluturhan et al., 2019; Zebral et al., 2020) can impact the biomarkers responses (Lomartire et  
85 al., 2021). It is necessary to make a distinction between the fluctuations of biological responses  
86 due to pollutants from the ones due to biotic or abiotic factors (Kalman et al., 2010).

87 Recently, several authors worked to define baseline data in biomarker using statistical analyses  
88 with the standardization of data and the implementation of a predictive model (Barbarin et al.,  
89 2022; Barrick et al., 2018; Baudou et al., 2021; Benito et al., 2019; Storhaug et al., 2019). The  
90 Convention for the Protection of the Marine Environment of the North-East Atlantic (OSPAR)  
91 and ICES (The International Council for the Exploration of the Sea) developed a method  
92 recommending the sampling of individuals at reference location over several seasons to record  
93 the variations of cofounding factors with the measure of biomarkers, and allowing the  
94 identification of polluted locations (Amiard-Triquet et al., 2015; OSPAR, 2013). It then defines

95 the range of values expected in a reference site as the baseline level and values outside this  
96 range could be classified into categories to define the range where environmental impacts are  
97 expected. Several approaches were tested to define these baselines, and demonstrated the need  
98 to use tools to identify anthropogenic and natural variations (Amiard-Triquet et al., 2015;  
99 OSPAR, 2013). It is also important to characterize the relationship between confounding biotic  
100 and abiotic factors to describe the influence of these parameters on the biomarker responses  
101 (Barrick et al., 2016). An effective approach is based on a multiple polynomial regression model  
102 and k-means clustering to standardize the influence of confounding factors on the biomarker  
103 response, but the model needs to be chosen carefully (Barbarin et al., 2022; Barrick et al., 2018;  
104 Burgeot et al., 2010).

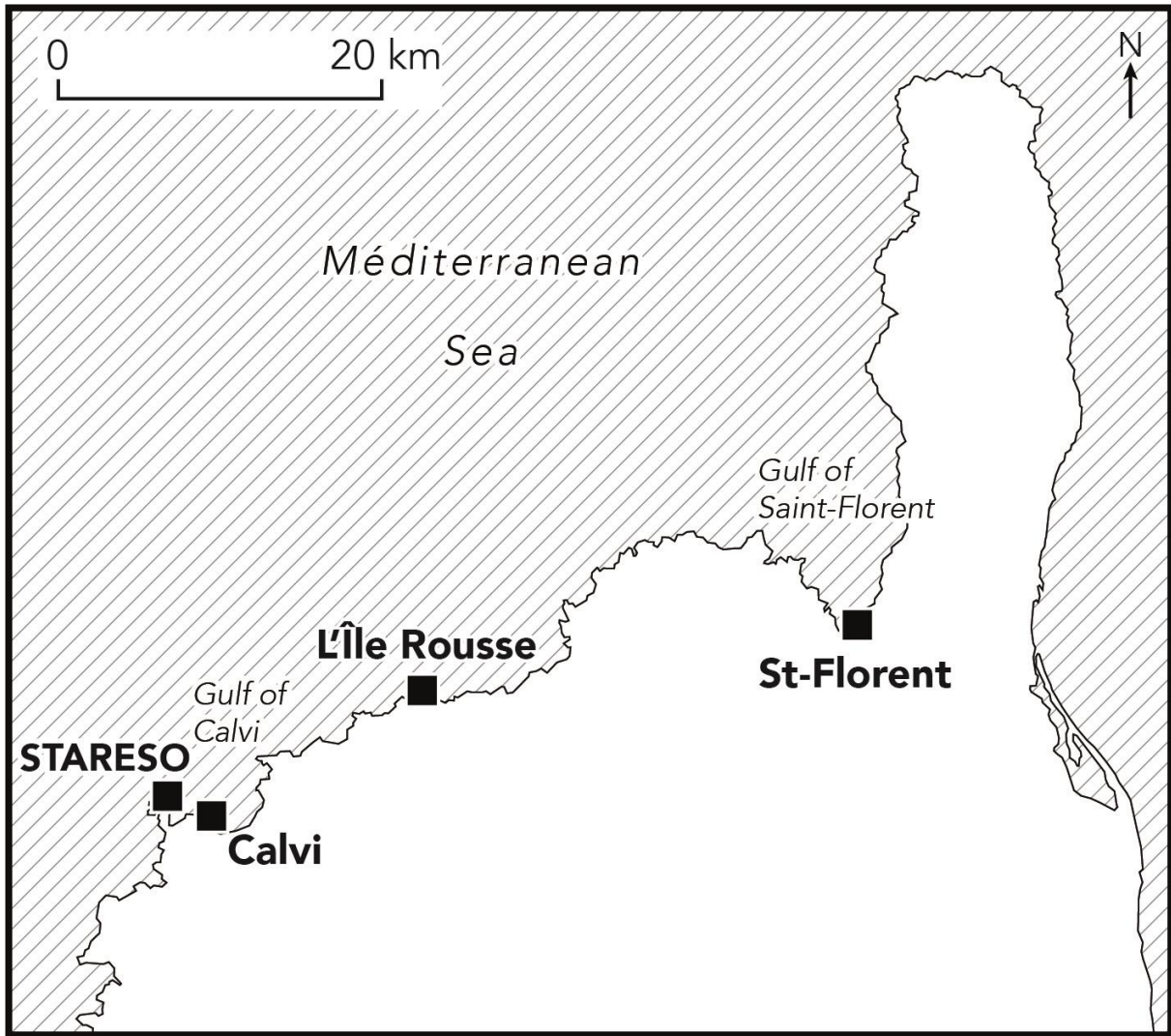
105

106 In the present study, the objective was to establish, through a predictive model, a baseline  
107 assessment criterion for biochemical biomarkers in limpet used for port area biomonitoring in  
108 North-Corsica. These additional data will provide more information on the ecological state of  
109 the ecosystem in this area and will be used to improve the environmental management of the  
110 ports.

111

112 St-Florent (N 42° 40' 50.362"; E 9° 17' 54.373"), Île Rousse (N 42° 38' 23.406"; E 8° 56' 7.778')  
113 and Calvi (N 42° 33' 59.116"; E 8° 45' 29.094") ports were chosen as exposed locations  
114 (Figure 1). The STARESO station in the Revellata bay (N 42° 34' 49.584"; E 8° 43' 31.511")  
115 was chosen as the reference location (Figure 1) because it is distant from any city.

116



117

118

**Figure 1** Map of the experimental locations in North Corsica (France).

119

120

At each sampling date, physicochemical parameter of water (temperature in °C and salinity in

121

PSU) were measured in the water column (~1 m of depth) using a CastAway CTD®

122

(Conductivity Temperature Depth) probe. Wild limpets ( $n > 7$  per location) were sampled

123

during two years (September 2020, January 2021, September 2021 and January 2022).

124

Seasonal, temperature and salinity, as well as the shell length of limpets collected at each

125

sampling period are shown in Table 1.

126

127 Table 1: Shell length (mean  $\pm$  SD) of collected limpet, temperature and salinity observed at  
 128 each sampling period for the different sampling locations

site	date	Sample			
		size	Shell length (cm)	Temperature ( $^{\circ}$ C)	Salinity (PSU)
STARESO	September 2020	8	4,06 $\pm$ 0,52	24,08	37,40
	January 2021	10	3,49 $\pm$ 0,36	13,65	37,74
	September 2021	10	3,7 $\pm$ 0,45	24,14	38,26
	January 2022	7	3,68 $\pm$ 0,44	14,12	38,45
Calvi	September 2020	8	4,05 $\pm$ 1,02	23,83	38,14
	January 2021	10	3,5 $\pm$ 0,4	13,26	37,47
	September 2021	8	3,7 $\pm$ 0,4	23,81	38,24
	January 2022	10	3,54 $\pm$ 0,46	13,24	38,34
Île Rousse	September 2020	8	3,3 $\pm$ 0,64	24,71	38,03
	January 2021	10	3,67 $\pm$ 0,31	12,60	37,75
	September 2021	10	3,29 $\pm$ 0,47	23,90	38,22
	January 2022	10	3,25 $\pm$ 0,4	13,33	38,44
St Florent	September 2020	8	3,22 $\pm$ 0,6	24,21	37,24
	January 2021	10	2,82 $\pm$ 0,2	11,39	29,08
	September 2021	10	3,35 $\pm$ 0,26	23,11	37,87
	January 2022	10	3,18 $\pm$ 0,38	13,86	37,36

129

130

131 Individuals were collected on site ( $n > 7$ , see Table 1), immediately frozen in liquid nitrogen  
 132 and kept at  $-80^{\circ}$ C until further biochemical analyses. Back in the laboratory, the whole soft  
 133 tissues of limpet were ground in liquid nitrogen using a MM400 Retsch<sup>®</sup> (GmbH, Éragny,  
 134 Luxemburg) mixer mill and then hand homogenized on ice in one volume of phosphate buffered  
 135 saline (PBS) solution (100 mM, pH 7.5) containing 0.1 % Triton X-100 and one mM  
 136 ethylenediaminetetraacetic acid (EDTA) to avoid protein degradation. The samples were  
 137 centrifuged 15 min at 12500 g at  $4^{\circ}$ C (Sorvall Legeng Micro 17R, ThermoFisher Scientific,  
 138 Waltham, Massachusetts, USA). The supernatant was used for the subsequent biomarker  
 139 analyses. The capacity to respond to oxidative stress was evaluated using superoxide dismutase  
 140 (SOD - EC 1.15.1.1) activity using Paoletti et al. (1986)'s protocol. The activity of glutathione  
 141 S-transferase (GST - EC 2.5.1.18), enzyme involved in detoxification process, was determined  
 142 following Habig et al. (1974). The laccase (LAC - EC 1.10.3.2) is involved in the immune,



143 antioxidant and detoxification processes (Luna-Acosta et al., 2017a). The activity of this  
144 enzyme was measured according to Luna-Acosta et al. (2010). The index of glycolysis  
145 gluconeogenesis pathway were determined with the pyruvate kinase activity (PK - EC 2.7.1.40)  
146 according to Childress and Somero (1979) as modified by Bailey et al. (2005).  
147 Acetylcholinesterase (ACHE - EC 3.1.1.7) is widely used to estimate neurotoxic impacts of  
148 pollutants, and its activity was assayed using Ellman et al. (1961)'s protocol. The total protein  
149 content was measured with the bicinchoninic acid protein assay kit (Sigma-Aldrich®) that is  
150 similar to the Lowry et al. (1951) procedure. Specific enzymatic activities were measured and  
151 expressed as U( $\mu\text{mol}\cdot\text{min}^{-1}$ ). $\text{mg}^{-1}$  of protein. All activities measurements were done on a  
152 SpectroStar Nano spectrophotometer (BMG labtech, Champigny-sur-Marne, France) in  
153 technical duplicate for each sample, using standard methods adapted for a microplate reader.  
154 All chemicals were obtained from Sigma-Aldrich®.

155 Statistical analyses were performed using R software version 4.2.0. Based on the analysed  
156 biomarkers (SOD, GST, LAC, PK and ACHE), data were organized by periods (4) and sites  
157 (4). Normality and homogeneity of variances were respectively verified by Shapiro and Levene  
158 tests. A series of observations were simulated according to mean values and standard deviation  
159 of each biomarker. Confounding factors (two physicochemical water parameters: temperature  
160 and salinity; and one biometric factor: shell length) and biomarker values of limpet collected in  
161 the reference location (STARESO) were used to determine formula describing the relationship  
162 between each biomarker and confounding factors (Press et al., 2007; Venables and Ripley,  
163 2013). The chosen cofactors were considered as the most relevant for the present study and are  
164 also often used in previous studies. Temperature and salinity were selected to consider the  
165 influence of environmental conditions (Almeida et al., 2022; Benito et al., 2019) and the shell  
166 length to consider the life stage of limpet.

167 Following this, pairwise analyses were conducted to identify which equation best described the  
168 relationship between each biomarker and the confounding factors. This established a  
169 polynomial regression formula. To address collinearity in the model, orthogonal polynomial  
170 regression was used to obtain new regression models with uncorrelated variables and achieved  
171 a final, stable polynomial regression equation for each biomarker:

172 **SOD (U.mg of protein<sup>-1</sup>)** = 868.59 + 36.88 (length) – 0.98 (length)<sup>2</sup> + 0.01(length)<sup>3</sup> – 166.3  
173 (temperature) + 4.36 (temperature)<sup>2</sup> + 4.17 (salinity)

174  $R^2 = 0.62$

175 **GST (mU.mg of protein<sup>-1</sup>)** = – 831.56 + 636.66 (length) – 16.79 (length)<sup>2</sup> + 0.15 (length)<sup>3</sup> –  
176 934.04 (temperature) + 24.1 (temperature)<sup>2</sup> + 42.1 (salinity)

177  $R^2 = 0.53$

178 **LAC (mU.mg of protein<sup>-1</sup>)** = 1.8 + 0.01 (length) – 0.02 (temperature) – 0.03 (salinity)

179  $R^2 = 0.31$

180 **PK (mU.mg of protein<sup>-1</sup>)** = 683.44 + 0.42 (length) – 0.77 (temperature) – 16.93 (salinity)

181  $R^2 = 0.21$

182 **ACHE (mU.mg of protein<sup>-1</sup>)** = 75.71 – 0.03 (length) – 0.34 (temperature) – 1.61 (salinity)

183  $R^2 = 0.24$

184

185 With these equations, the predicted values were calculated for the reference location. The  
186 residuals were obtained by subtracting the predicted values from the values obtained in the  
187 laboratory. Using k-means clustering (Barrick et al., 2016; Barrick et al., 2018), the residuals  
188 were classified into three groups that were used to determine the range of variations according  
189 to environmental conditions and to define the baseline level (Table 2).

190

191 Table 2: Level of traffic light scheme (red: lethal effects expected, orange: sublethal effects  
192 expected, yellow: range expected on non-impacted sites, green: evidence of good health) used

193 to define the range of environmental effects. Y indicates the mean value for the reference site  
 194 (STARESO). SOD (superoxide dismutase), GST (gluthathione-S-transferase), LAC (laccase  
 195 type phenoloxidase), PK (pyruvate kinase), ACHE (acetylcholinesterase).

Indicators	SOD residuals	GST residuals	LAC residuals	PK residuals	ACHE residuals
Green	< y - 33.21 [y - 33.21; y +	< y - 149.61 [y - 149.61; y +	< y - 0.30 [y - 0.30; y +	< y - 20.85 [y - 20.85; y +	< y - 3.69 [y - 3.69; y +
Yellow	83.68] [y + 83.68; y +	320.25] [y + 320.25; y +	0.20] [y + 0.20; y +	13.21] [y + 13.21; y +	2.36] [y + 2.36; y +
Orange	90.43]	344.56]	0.54]	169.99]	20.12]
Red	> y + 90.43	> y + 344.56	> y + 0.54	> y + 169.99	> y + 20.12

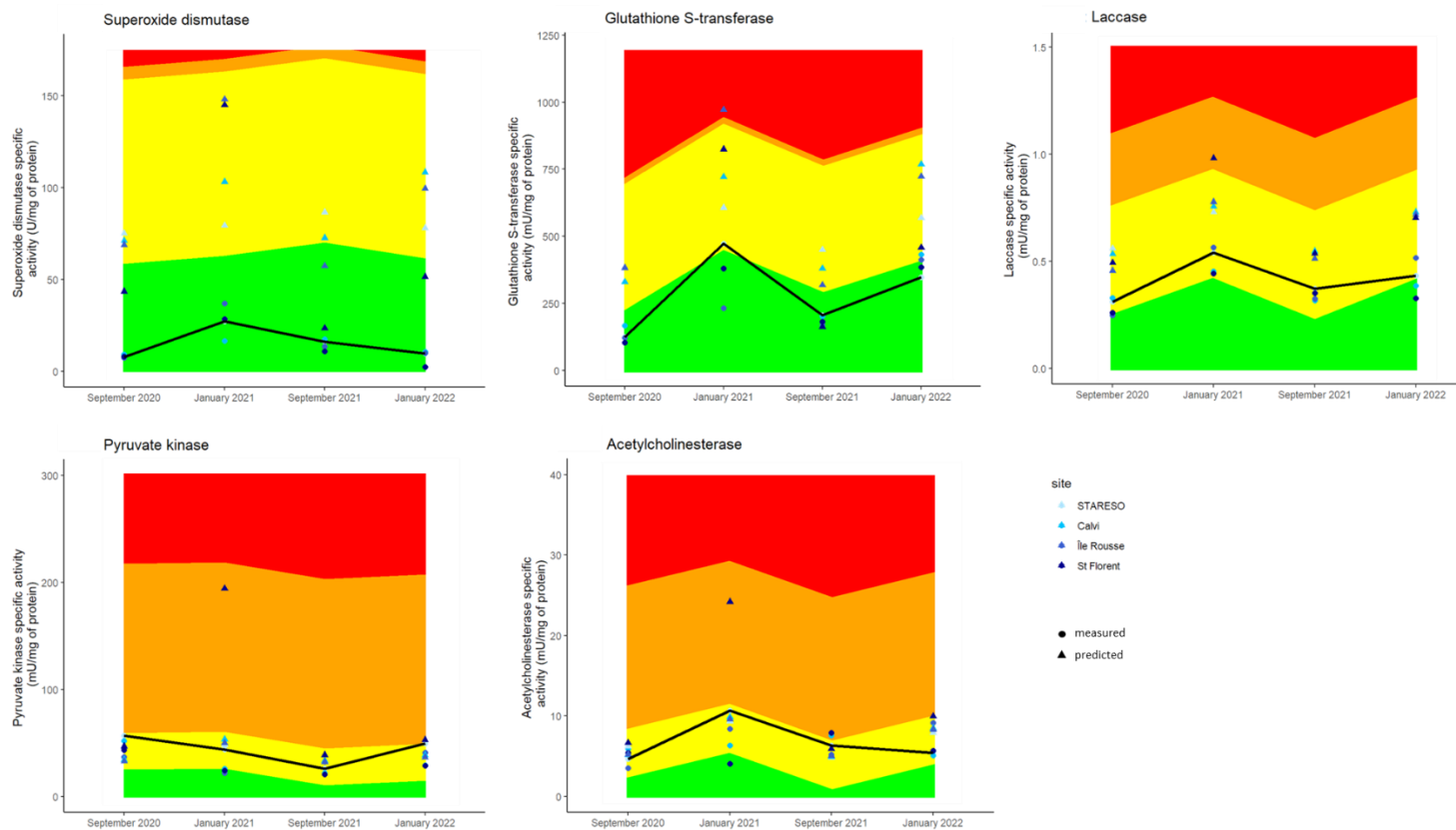
196  
 197 The equations were then used to calculate the predicted value for each biomarker on each  
 198 location. These values and the average biomarker activity for the reference location (black line)  
 199 were graphically represented within the range of variation for each biomarker (Figure 2). The  
 200 interpretation of the data should be taken with precaution, because statistical analyses  
 201 considering maximum of variable is always complex to explain and error of interpretation can  
 202 occur. However, for environmental studies, it is essential to consider the influence of  
 203 environmental parameters with time and location. It is also essential to take into account  
 204 morphometric parameter of the individual as they are often collected as wild in the environment.

205  
 206 The model displayed the seasonal variations of the biomarker baseline. In general, the activity  
 207 of the biomarkers is lower in winter. This winter decrease is consistent with previous studies  
 208 results (Barbarin et al., 2022; Barrick et al., 2018; Storhaug et al., 2019) and could be explain  
 209 by several factors. The lower temperatures in winter could induce a slower metabolism and  
 210 subsequently lower enzymes activities. Seasonal variations in could also be related to the  
 211 reproductive state of organisms as it was observed for mussels by Storhaug et al. (2019). Having  
 212 more information on the seasonal variations of biomarkers could help to improve biomonitoring  
 213 of specific environments such as ports areas. In the ports selected in the present study, the  
 214 decrease observed in winter should be considered. It would be necessary to obtain a more

215 precise view of the variation along the year by collecting additional samples during the other  
216 period of the year and to do so for a longer period of time. After that, biomonitoring of the port  
217 area should be realized during months that are comparable to each other (with the same  
218 baseline) to observe the impact of pollution and not the natural variation of biomarkers.

219

220 The results showed that the observed values for all the biomarkers are very close to the value  
221 observed for the reference site (STARESO) and fell within the green or yellow ranges, showing  
222 either evidence of good health or unimpacted organisms (Figure 2). For PK and ACHE, the  
223 values predicted by the model were also really close to the observed values. This reflects good  
224 predictions of the model for these two biomarkers. The only exceptions for PK and ACHE are  
225 the values predicted at St-Florent in January 2021. This value is much higher than the other  
226 ones and fell between the orange range where sublethal effects are expected. For the SOD, GST  
227 and LAC, the observed values also fell pretty close to the value observed for the reference site,  
228 so it is still a good sign for the ecological status of the organisms in North Corsica ports.  
229 However, the difference between observed and predicted values are more important for these  
230 three biomarkers. Despite this difference, the observed value fell most of the time within the  
231 yellow range where no impacts should be detected on organism. In January 2021, the predicted  
232 values for LAC in St-Florent and GST in Île Rousse are the exceptions, falling respectively in  
233 the orange (sublethal effects expected) and red range (lethal effects expected). The difference  
234 between observed and predicted values for SOD, GST and LAC is slightly more important in  
235 winter, especially in January 2021.



236

237 **Figure 2** Graphical representation of health indicator (red: lethal effects expected, orange: sublethal effects expected, yellow: range expected on  
 238 non-impacted sites, green: evidence of good health) for superoxide dismutase, glutathione-S-transferase, laccase type phenoloxidase, pyruvate  
 239 kinase and acetylcholinesterase in STARESO (reference location), Calvi, Île Rousse and St Florent. For each location, the observed and predicted  
 240 (from the equations defined in the present study) values are respectively represented by circles and triangles. The black line represents the  
 241 observed value for the reference location (STARESO).

242 The statistical model works pretty well. For most of the data and especially for PK and ACHE,  
243 the predicted values are consistent with the observed ones. These results confirm that the  
244 equations of the model were correctly selected. However, the model still needs some  
245 improvements for the SOD, LAC and GST, and this is especially true for the winter periods  
246 (2021 and 2022) and the St-Florent port. The different results between the observed values and  
247 the model predictions could be explained by several possibilities. The choice of cofounding  
248 factors incorporated into the model can influence the results. The environmental factors  
249 included in the present model are the most common in similar studies (Barbarin et al., 2022;  
250 Barrick et al., 2016; Barrick et al., 2018) but can also change to a more or less extent from one  
251 year to another. However, further investigations are necessary to identify what else can  
252 influence the biomarkers values (nutrient availability, reproductive status, life stage, light,  
253 presence of contaminants, etc.) (Barrick et al., 2016; Barrick et al., 2018). For example, St-  
254 Florent port is characterized by the presence of the Aliso River mouth. A higher input of  
255 freshwater in this port probably has an influence on the biomarker responses and should be  
256 taken into consideration in a future model.

257 Additional factors could be easily incorporated into the model but it will be necessary to  
258 evaluate the covariation between these factors. This can be rather difficult as some of these can  
259 be strongly linked (e.g. nutrient availability and light, life stage and weight, etc.) (Barrick et al.,  
260 2018). Once the characterization of the cofactor's correlation included into the model, it can be  
261 used discrimination between cofounding factors and to identify the impacts of contaminants.  
262 For futures studies and to improve the precision of the model, it will be important to collect  
263 more organisms to increase the number of samples and to run the analysis for a longer time  
264 period to consider the variations between years.

265

266 The objective of this study was to design a baseline level for biomarkers that account for  
267 confounding factors. By providing more data on the ecological status of port areas, the  
268 development of this model for limpets will help the implementation of biomonitoring  
269 environmental program in North Corsica ports. Already developed for ragworms (Barrick et  
270 al., 2016; Barrick et al., 2018), bivalve (Barbarin et al., 2022; Storhaug et al., 2019) or fish  
271 (Baudou et al., 2021), this kind of statistical approach provide a better understanding of the  
272 biomarkers responses. The present study is the first to develop this model on limpet. It also  
273 brings more data for the use of this specie in biomonitoring program, as it was shown to be a  
274 promising bioindicator in previous studies (Mbandzi et al., 2022; Pérez et al., 2019; Sánchez-  
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276

277

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455 **Statements and declarations**

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461

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463 The authors have no relevant financial or non-financial interests to disclose.

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465

466 **Authors Contributions**

467 Marion Pillet: conceptualization, methodology, software, validation, data collection, formal  
468 analyses, investigation, resources, writing, visualization, project administration.

469 Frédéric Muttin: conceptualization, methodology, software, validation, formal analysis,  
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471 Michel Marengo: data collection, project administration, funding acquisition

472 Lovina Fullgrabe: data collection, formal analysis.

473 Valérie Huet: formal analyses.

474 Pierre Lejeune: conceptualization, funding acquisition.

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476

477 **Ethical Approval**

478 This study does not involve human participants or vertebrate animals.

479

480 **Consent to Participate**

481 Yes

482

483 **Consent to Publish**

484 Yes

485

486 **Availability of data and materials**

487 The datasets generated during and/or analysed during the current study are available from the  
488 corresponding author on reasonable request.

489

490 List of captions:

491 **Fig. 1** Map of the experimental locations in North Corsica (France)

492

493 **Fig. 2** Graphical representation of health indicator (red: lethal effects expected, orange:  
494 sublethal effects expected, yellow: range expected on non-impacted sites, green: evidence of  
495 good health) for superoxide dismutase, glutathione-S-transferase, laccase type phenoloxidase,  
496 pyruvate kinase and acetylcholinesterase in STARESO (reference location), Calvi, Île Rousse  
497 and St Florent. For each location, the observed and predicted (from the equations defined in the  
498 present study) values are respectively represented by circles and triangles. The black line  
499 represents the observed value for the reference location (STARESO).

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501