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1 **First insight into the development of a new transcriptomic tool in French Corsica harbors**

2

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16

17 **Abstract**

18 Coastal harbor areas are subjected to a myriad of pollution sources with largely unknown
19 effects. Such complex chemical mixtures are difficult to monitor but transcriptomics is a
20 promising approach for such biomonitoring. The present study was designed to verify the use
21 of the Coastal Biosensor for Endocrine Disruption (C-BED) assay, previously developed to
22 detect emerging contaminants and their effects on *Mytilus edulis*, on another mussel species,
23 *Mytilus galloprovincialis*. Mussels were caged on St-Florent harbor (contaminated) and on
24 Revellata Bay (reference) for three months. A classical multibiomarkers approach was coupled
25 to the C-BED assay. The results of both approaches were analysed using the Integrated
26 Biomarkers Responses (IBR) and compared to each other. Both approaches demonstrated a
27 higher contamination and probable endocrine disruption of mussels in St-Florent, compared to
28 the reference station. These results confirm that the C-BED assay provides an innovative
29 method to expands our ability to detect emerging contaminants.

30

31 Keywords: chronic pollution, emergent contaminant, C-BED, biomonitoring, *M.*
32 *galloprovincialis*, multibiomarkers.

33

34 **Introduction**

35 Coastal harbor areas are susceptible chronic and diffuse marine pollution due to contaminant
36 run-off from natural and anthropogenic sources (e.g. terrestrial discharges, anthropogenic
37 contaminations) and estuarine characteristics (e.g., low hydrodynamism) (Islam and Tanaka,
38 2004). This pollution is a serious threat to the marine environment and recreational activities
39 and could pose a subsequent risk to human health (Maisano et al., 2017). Recently, the presence
40 of legacy pollutants (trace elements, PAHs, PCBs) has declined within urban coastal systems,
41 but emerging contaminants (e.g. pharmaceuticals, perfluorinated alkylated substances,

42 nanomaterials) are becoming more prominent even has their impacts are not fully understood
43 (Maruya et al., 2014; Richardson, 2009). The concern about these compounds has increased
44 due to endocrine disruptive effect and their potential to cause population level effects in wildlife
45 at low exposure levels (Blalock et al., 2020). In addition, these chemicals are frequently found
46 together in complex mixtures. Determining the impacts of emerging contaminants and how they
47 interact with other chemical stressors to cause additive or synergistic effects is one of the major
48 challenges in ecotoxicology today (Van den Brink et al., 2018).

49 In 2008, the European Commission approved the Marine Strategy Framework Directive
50 (DCSMM, 2008), a legislation aimed at achieving or maintaining “Good Environmental Status”
51 in EU waters. In North Corsica, to our knowledge, no study has been done within its harbor
52 areas, although it is important to evaluate and monitor the quality of the waters and the
53 ecosystem.

54 Marine biomonitoring consists in observing and assessing the natural or induced
55 alterations of marine ecosystem and this evaluation can be measured at several levels, from the
56 molecular, cellular, and individual levels to the populations up to whole biocenosis level
57 (Bondaruk et al., 2015). Bivalves and especially mussels have been used as bioindicators for
58 decades to investigate the toxicity of pollutants. The classical example of biomonitoring is the
59 Mussel Watch concept, which was initiated in 1976, consisting in the surveillance of United
60 States coastal regions for their pollutant contents (Farrington et al., 2016). The use of these
61 bioindicators is particularly relevant from a biological point of view as the directly measured
62 concentrations reflect the bioavailable fractions in the marine environment (Wang, 2016).
63 Mussels are ideal for biomonitoring studies because they are filter feeders, are distributed
64 worldwide, and have capacity to bioaccumulate diverse contaminants (Banni et al., 2016;
65 Canesi et al., 2008; Ozkan et al., 2017; Strehse and Maser, 2020). They also provide several

66 biomarker responses, such as decreased antioxidant defences in the presence of pollution (Beyer
67 et al., 2017).

68 Despite the sensitivity of conventional biomarkers for biomonitoring and risk
69 assessment (Handy et al., 2012), these indicators may not respond to low level, chronic
70 exposures to pollutants or exposure to emergent pollutants. More integrative tools, are needed
71 that offer greater sensitivity and specificity across diverse pollutant classes (Gottschalk et al.,
72 2013). Transcriptomic and other High Throughput Screening (HTS) approaches, are of great
73 interest because they allow the screening of a large set of genes and the impact of pollutants on
74 their expression (Châtel et al., 2018; Poynton and Vulpe, 2009).

75 In the present study, a classic multi-biomarker approach was coupled to an innovative
76 transcriptomic approach that uses the biological responses of blue mussels to detect the additive
77 impacts of complex contaminant mixtures found in urban coastal areas. The Coastal Biosensor
78 for Endocrine Disruption (C-BED), was designed based the responses of *Mytilus edulis* exposed
79 to estrogenic compounds in the lab, and was successfully validated by detecting endocrine
80 disrupting compounds (EDCs) in field collected mussels in Northeastern U.S.A. (Blalock et al.,
81 2018; Blalock et al., 2020). The assay uses the transcriptomic responses of a specific set of
82 biomarker genes chosen because of their roles in endocrine pathways and sexual development,
83 and their robust responses to endocrine disrupting stressors.

84 The goal of this paper is to translate this innovative method to a different mussel species, *M.*
85 *galloprovincialis*, and to test the incorporation of a molecular tool in a classic biomonitoring in
86 Mediterranean harbors of North Corsica.

87

88 **Material and methods**

89 Caged mussels (*M. galloprovincialis*) were deployed in St. Florent harbor (exposed site, North
90 Corsica, N 42° 40' 50.362"; E 9° 17' 54.373") and in the Revellata Bay (control site, N 42° 34'

91 49.584"; E 8° 43' 31.511") for three months before sampling in September 2020 and January
92 2021. At the end of the exposure, 20 mussels were collected for subsequent classic biomarkers
93 (n = 10) and molecular (C-BED, n = 10) analyses.

94

95 **A. Classic biomarker approach**

96 Individuals collected for the classic biomarkers were immediately placed in dry ice, shipped to
97 the laboratory within 48 h and kept at -80°C until enzymatic analyses.

98 The digestive gland of mussel was dissected and ground in liquid nitrogen using a MM400
99 Retsch© (GmbH, Éragny, Luxemburg) mixer mill and homogenized on ice in phosphate
100 buffered saline (PBS) solution (100 mM, pH 7.5) containing 0.1 % Triton X-100 and 1 mM
101 methylene diamine tetra-acetic acid (EDTA) to avoid protein degradation. The samples were
102 centrifuged, and supernatant was collected for the subsequent biomarkers of effect and protein
103 analyses.

104 A multibiomarkers approach was used by testing superoxide dismutase (SOD), catalase (CAT),
105 glutathione peroxidase (GPx), glutathione S-transferase (GST), pyruvate kinase (PK),
106 acetylcholinesterase (AChE) and laccase-type phenoloxidase (LAC). SOD activity protocol is
107 based on the inhibition of a superoxide-driven NADH oxidation and was measured following
108 the rate of NADH oxidation at 340 nm (Paoletti et al., 1986). CAT activity was determined
109 according to Aebi (1984) by measuring the rate of decomposition of hydrogen peroxide at 240
110 nm. GPx activity was assayed according to Paglia and Valentine (1967) as modified by Janssens
111 et al. (2000) by monitoring the consumption of NADPH at 340 nm. GST activity was
112 determined by measuring the production of GS-DNB at 340 nm (Habig et al., 1974). The
113 activity of PK was determined by measuring the NADH consumption at 340 nm, according to
114 Childress and Somero (1979) as modified by Bailey et al. (2005). AChE activity was assayed
115 by recording the production of 5-thio-2-nitrobenzoate at 405 nm (Ellman et al., 1961). Finally,

116 the LAC activity was measured according to Luna-Acosta et al. (2010), following the oxidation
117 of phenylenediamine at 420 nm. The total protein content was measured with the bicinchoninic
118 acid protein assay kit (Sigma-Aldrich®) that is similar to the Lowry et al. (1951) procedure.
119 All activities measurements were done on a SpectroStar Nano spectrophotometer (BMG
120 labtech, Champigny-sur-Marne, France). Chemicals were obtained from Sigma-Aldrich®. All
121 analyses were performed in duplicate using standard methods adapted for a microplate reader.
122 For all enzymatic analysis, homogenates were diluted to obtain linear reaction slopes for a
123 minimum of five minutes at 25 °C. Specific enzymatic activities were measured and expressed
124 as U ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$) of protein.

125

126 **B. Transcriptomic approach**

127 For the C-BED assay, mussel ($n = 10$ for each period) were immediately dissected and
128 the digestive gland was kept in RNAlater. Total RNA was extracted using TriReagent
129 (Molecular Research Center, Cincinnati, OH), according to the manufacturer's protocol.
130 Samples were then treated with DNase (New England Biolabs, Ipswich, MA) to remove any
131 residual DNA. Samples were cleaned up using a NEB Monarch RNA Cleanup Kit (New
132 England Biolabs) to purify the RNA samples. RNA was reverse transcribed to cDNA using
133 EpiScript Reverse Transcriptase (Lucigen, Middleton, WI) (Blalock et al., 2018; Blalock et al.,
134 2020; Hines et al., 2007).

135 The C-BED assay uses real time quantitative PCR (RT-qPCR) and consists of eleven genes:
136 two sex marker genes (for sex identification: VERL, VCL), eight biomarker genes (Cav-1,
137 StAR, MBTP, BCAS3, SR, ADX, GCL32E, Mat89bb), and one reference gene (18S) (Blalock
138 et al., 2018; Blalock et al., 2020). *M. edulis* primers sequences from Blalock et al. (2018) were
139 tested on the *M. galloprovincialis* samples and verified on an agarose gel. All primers, except
140 VERL, produced a product. New primers for *M. galloprovincialis* VERL (GenBank:

141 VDI63802.1) were designed using Primer3 (v4.1.0) (F: GCT GTT GCG TTG CTA TCA CT
142 R: TGC AGA TGA CGC CTT TAT GC) and were tested and verified using qPCR.
143 All qPCR reactions were performed as described previously (Blalock et al., 2018) using
144 PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, Waltham, MA). Standard
145 curves for all primers, including the newly designed *M. g.*, VERL primers, used had an r^2 of
146 0.99 and a primer efficiency between 95-105% (Blalock et al., 2018). The difference in
147 expression in VERL and VCL was used for sex identification of *M. galloprovincialis* mussels
148 (Blalock et al., 2018; Hines et al., 2007). A difference in Ct greater than 2.5 indicates the mussel
149 is a male, less than -2.5 is a female, and between -2.5 and 2.5 is unknown (i.e., sex cannot be
150 determined) (Blalock et al., 2018; Hines et al., 2007). Expression of the eight biomarker genes
151 were normalized to 18S expression. The C-BED assay was performed on all mussels including
152 those identified as male, female, or undetermined. This is in contrast with our previous study
153 using the C-BED assay, which limited assay responses to male mussels only (Blalock et al.,
154 2018). We have decided to extend the assay to all mussels because (1) there was limited
155 difference between the IBR score of males vs females in this study (see supplement) and (2) in
156 the presence of endocrine disrupting compounds, male mussels may become phenotypically
157 female and be identified as female mussels (Evensen in prep). Therefore, focusing only on
158 mussels identified as male following an exposure is not including the responses of all
159 genetically male mussels.

160

161 **C. Statistics**

162 Data were compiled into the Integrated Biomarker Response (IBR). IBR was developed by
163 (Beliaeff and Burgeot, 2002). The IBR was described to be useful in environmental quality
164 assessment to evaluate the impact of environmental conditions on the health of organisms and
165 their physiological status (Serafim et al., 2012) and was shown to be useful to distinguish among

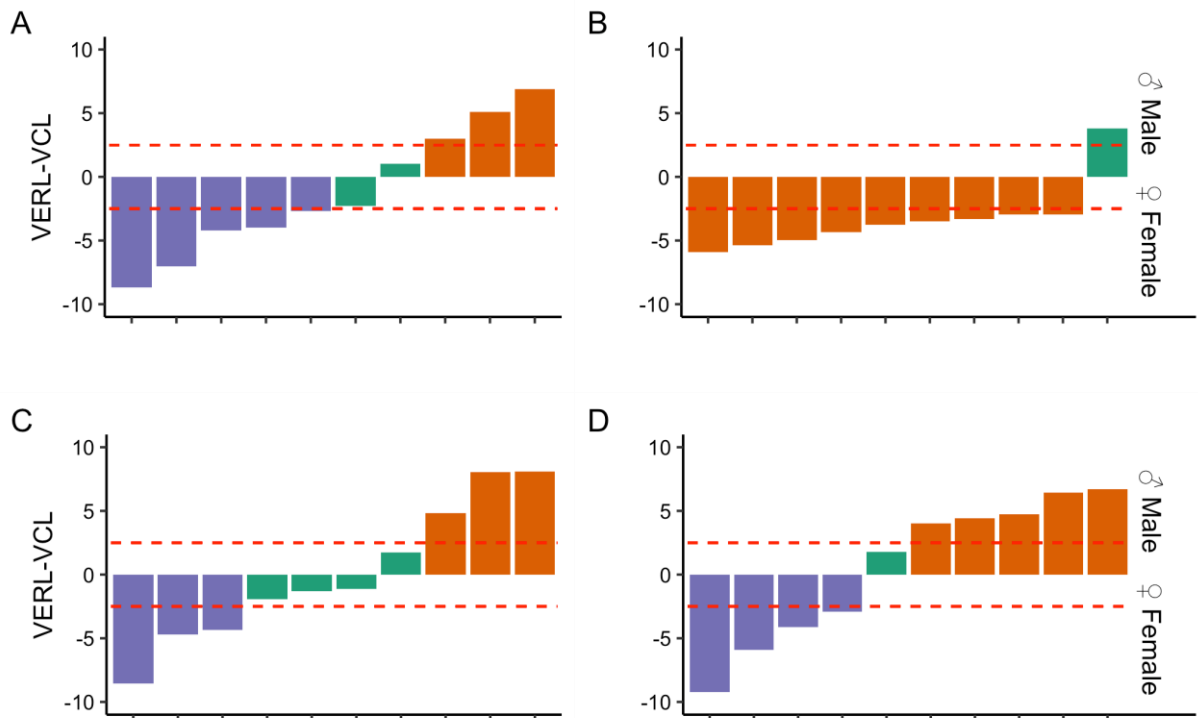
166 sites located along a stress gradient, while individual biomarkers were not able to do so
167 (Bertrand et al., 2018). Devin et al. (2014) adjusted the IBR calculation by determining every
168 permutation of the IBR based on different positions of the biomarkers and calculating the
169 median IBR value. The median IBR value was calculated for both sites at both time points. On
170 these data, normality and homogeneity of variances were verified by Shapiro and Levene tests
171 and data. As these conditions were not found and no data transformations was possible, non-
172 parametric Kruskal-Wallis tests, were performed to analyse the effect of treatment, followed
173 by Mann-Whitney Wilcoxon post-hoc test when a significant effect was found.

174

175 **Results**

176 Mussel sex identification was performed to evaluate for skewed sex ratios. There was no
177 significantly skewed sex ratio at Revellata Bay at either time points or at St. Florent January
178 2021. There was a significantly skew sex ratio ($p < 0.05$) at St. Florent in September 2021
179 (**Figure 1**). At St. Florent in September, there was only one male out of the ten sampled mussels;
180 the remaining mussels were all female.

181



182

183 Figure 1: Sex identification results at Revellata Bay and St. Florent. Bars greater than 2.5 are
 184 male, and bars less than -2.5 are female. Bars that are between -2.5 and 2.5 indicate mussels
 185 whose sex could not be determined. A) Revellata Bay, September 2020. B) Revellata Bay,
 186 January 2021. C) St Florent, September 2020. D) St. Florent, January 2021.

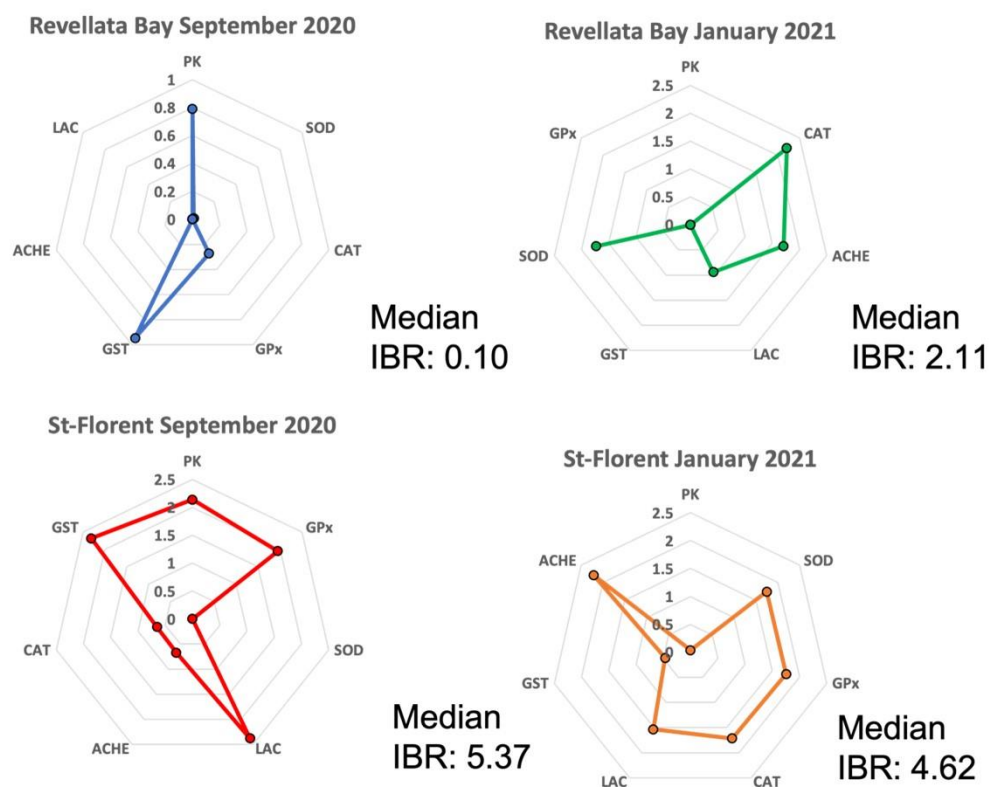
187

188

189 The classical biomarkers showed higher IBR values (figure 2) at St-Florent (median =
 190 5.37 in September and 4.62 in January) compared to Revellata Bay (median = 0.105 in
 191 September and 2.11 in January), and a greater difference in the summer.

192 The relative importance of each biomarker varies according to season and site: in St-Florent in
 193 September, all enzymes present relatively low coefficients, while the higher coefficients are
 194 observed for LAC, GPx, GST and PK in St-Florent in January and for CAT, ACHE and SOD
 195 in Revellata Bay at both seasons.

196



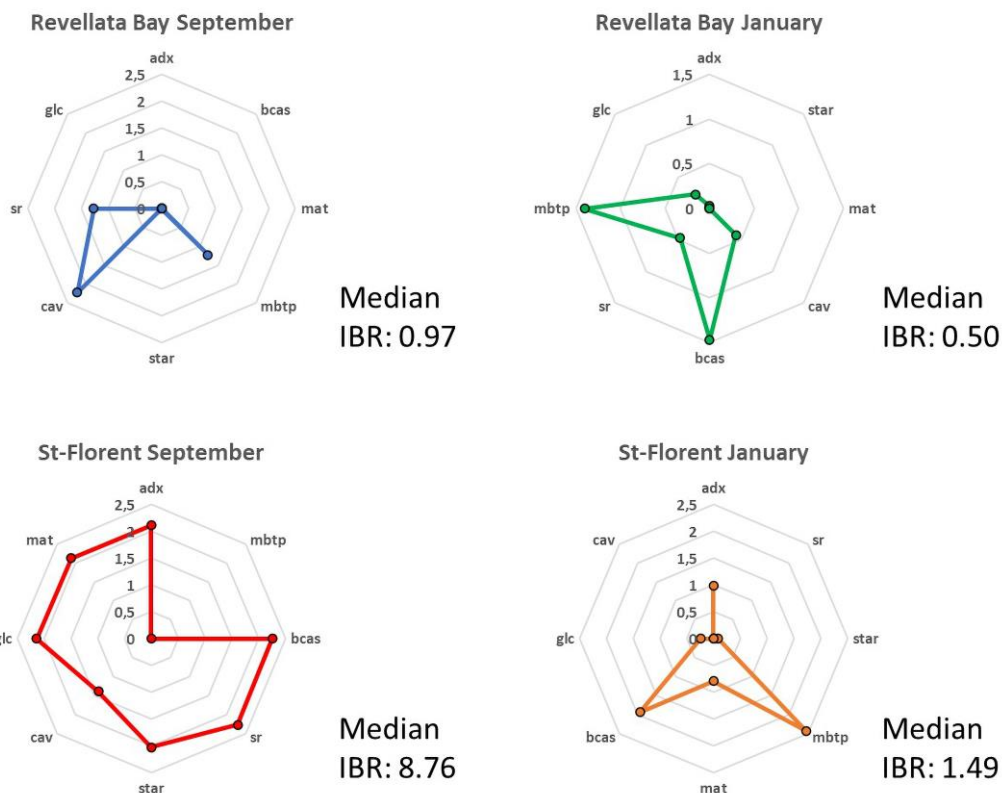
197

198 Figure 2: Median IBR values calculated using data from the classical biomonitoring data

199

200 Similarly to the classical biomarkers, the C-BED assay showed higher IBR values at St.
 201 Florent compared with Revellata Bay (**Figure 3**). At both time points, the IBR values at St.
 202 Florent were higher than Revellata Bay. In September 2020 and January 2021, Revellata Bay
 203 had IBR values less than 1, while St-Florent had IBR values greater than 1, reaching 8.76 in
 204 September 2020 (**Figure 3**). At both sites, the IBR values for January were lower than those for
 205 September (**Figure 3**). Based on the IBR values, we ranked the sites and time points based on
 206 risk of endocrine disruption: Revellata Bay January 2021 < Revellata Bay September 2020 <
 207 St. Florent January 2021 < St. Florent September 2020.

208



209
 210 Figure 3: Median IBR values for Revellata Bay (September 2020, January 2021) and St Florent
 211 (September 2020, January 2021) for the C-BED Assay. The position of the biomarkers is
 212 adjusted for each condition to reach the median IBR value

213
 214 **Discussion**

215 *Mussels at St. Florent are exposed to a complex mixture*
 216 The C-BED assay and the other biomarkers indicate that *M. galloprovincialis* at St. Florent are
 217 exposed to a complex mixture of contaminants, including EDCs. The higher classic biomarker
 218 IBR values at St. Florent compared to Revellata Bay (**Figure 2**) indicate there are contaminants
 219 in the water, causing a stress response in the St. Florent mussels. These results confirm
 220 observations described in previous environmental biomonitoring studies. St-Florent is usually
 221 described as under moderate anthropogenic pressure (Gobert et al., 2009) or even as a highly
 222 contaminated site (Ternengo et al., 2018). The contamination is usually ascribed to high levels

223 of metallic trace elements such as Co, Cr, Fe or Ni, linked to waste from the former Canari
224 asbestos mine, closed in 1965, located at the northwest of the Gulf of St-Florent (Andral et al.,
225 2004; Luy et al., 2012; Ternengo et al., 2018). However, there is little data available on pollution
226 levels in this region, especially for organic contaminants.

227

228 *C-BED assay is translatable across Mytilus species*

229 Here, we adapted the C-BED assay, which was originally developed in *M. edulis* (Blalock et
230 al., 2018), to the closely related *M. galloprovincialis*. This assay is used as an early indicator
231 for possible developmental and reproductive impairments. There was a higher median IBR
232 value at both time points at St. Florent (Sept: 8.79, Jan: 1.49) than at Revellata Bay (**Figure 3**).
233 The results of the C-BED assay indicate that mussels at St. Florent are responding to EDCs.
234 Furthermore, the significantly feminized sex ratio ($p < 0.05$) at Revellata Bay in September
235 (**Figure 3C**), during the same sampling period as the highest IBR values, strongly suggests that
236 endocrine disruption is occurring in these mussels. In addition, the difference in IBR values at
237 the impacted and reference site show that this assay can be applied to *M. galloprovincialis*. The
238 *Mytilus* species complex are found globally, from South America to the North Atlantic to the
239 Pacific (Oyarzún et al., 2021; Wilson et al., 2018). With the success of application of the C-
240 BED assay to *M. galloprovincialis*, this assay can be utilized in other *Mytilus* species.

241

242 *Benefits of a multi-biomarker approach*

243 Using IBR values from both approaches indicates mussels are exposed to a variety of
244 contaminants including contaminants that elicit classic stress responses and those that cause
245 endocrine disruption. Interestingly, both approaches indicated that contamination levels and
246 effects are more severe during the summer. These effects might be due to the higher touristic
247 activity in summer or to the elevated temperature for example. A single compound at a low

248 concentration may not produce a detectable adverse effect in an organism. However, many
249 contaminants at low concentrations may cause additive impacts, resulting in a detectable effect
250 (Giuliani et al., 2013; Strömgren, 1986; Widdows and Donkin, 1992). Adding a transcriptomic
251 assay to biomonitoring can provide vital information because gene expression changes are
252 highly responsive and sensitive (Aymoz et al., 2018; Bendjilali et al., 2017). Furthermore,
253 utilizing multiple assays and IBR values provides greater insight to possible exposures and
254 stress responses organisms may be experiencing (Beliaeff and Burgeot, 2002; Devin et al.,
255 2014; Giuliani et al., 2013; Kim et al., 2014).

256

257 **Conclusion**

258 Here we demonstrated that the C-BED assay is translatable to a new *Mytilus* species,
259 broadening its application and that its inclusion within a multi-biomarker monitoring program
260 provides insight into the chemicals present and causing biological effects within urban harbors.
261 Determining the impacts of emerging contaminants and how they interact with other chemical
262 stressors to cause additive or synergistic effects is one of the major challenges in ecotoxicology
263 today. The global application of the C-BED assay addresses this challenge and will significantly
264 enhance our ability to predict environmental impacts of contamination found in our urban
265 coastal ecosystems and harbors. Through this, we anticipate the development of a
266 transformative tool for coastal environmental assessment into management decisions for coastal
267 areas.

268

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272

274 **Authors Contributions**

275 Marion Pillet: conceptualization, methodology, validation, data collection, formal analyses,
276 investigation, writing.

277 K. Garrett Evensen: methodology, validation, data collection, formal analyses, investigation,
278 writing.

279 Michel Marengo: conceptualization, methodology, supervision, project administration, funding
280 acquisition.

281 Pierre Lejeune: conceptualization, funding acquisition.

282 Helen C. Poynton: conceptualization, supervision, funding acquisition.

283 H el ene Thomas: supervision, project administration, funding acquisition.

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290

291 **Competing Interests**

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

293 The authors have no competing interests to declare that are relevant to the content of this article.

294

295 **Ethical Approval**

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

297

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441 Figure 4: Sex identification results at Revellata Bay and St. Florent. Bars greater than 2.5 are
442 male, and bars less than -2.5 are female. Bars that are between -2.5 and 2.5 indicate mussels
443 whose sex could not be determined. A) Revellata Bay, September 2020. B) Revellata Bay,
444 January 2021. C) St Florent, September 2020. D) St. Florent, January 2021.

445 Figure 5: Median IBR values calculated using data from the classical biomonitoring data

446 Figure 6: Median IBR values for Revellata Bay (September 2020, January 2021) and St Florent
447 (September 2020, January 2021) for the C-BED Assay. The position of the biomarkers is
448 adjusted for each condition to reach the median IBR value

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