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Investigating the impact of remediation efforts on *Hediste diversicolor* in the Seine estuary using multiple levels of biological organization

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1 **Investigating the Impact of Remediation Efforts on *Hediste diversicolor* in the Seine**
2 **Estuary Using Multiple Levels of Biological Organization**

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14
15 **Abstract**

16 The Seine Estuary is historically one of the most contaminated estuaries in Europe. In 2002 the
17 estuary underwent major redevelopment which emphasized environmental remediation to measure
18 biological effects. The present study investigated the health status of the sentinel species *Hediste*
19 *diversicolor* in the Seine estuary by comparing data from a sampling campaign immediately after the
20 Seine underwent reconstruction and 10 years afterward. Both studies implemented multiple levels of
21 biological organization ranging from enzymatic biomarkers to population density. Integrative
22 modeling was used to establish a holistic status assessment for *H. diversicolor* in the Seine. *H.*
23 *diversicolor* populations in the Seine estuary were impacted in both campaigns suggesting no
24 significant improvements over the 10-year period. This interpretation however can be improved by
25 increasing the number of reference sites used to establish the baseline natural variation, integrating
26 additional environmental parameters into the model and measuring additional biological endpoints,
27 such as reproductive status.

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39 **Introduction**

40 The Seine Basin in France represents a catchment area of approximately 78,650 km² and
41 encompasses 40% of the French economic activity including intensive agricultural activities, strong
42 industrialization, and significant urbanization (Billen et al., 2007). Due to a long history of human
43 activity, the Seine estuary is considered one of the most contaminated water systems in Europe with
44 a wide variety of persistent pollutants in the environment including metals, polycyclic aromatic
45 hydrocarbons (PAHs), and polychlorinated biphenyls (PCBs) (Blanchard et al., 2007; Hamzeh et al.,
46 2014; Thévenot et al., 2007). This results in a complex mixture of anthropogenic contamination that
47 arises in short-term and long-term impacts on ecosystem health and makes effective mitigation
48 difficult. The Seine estuary is a major water system that is crucial for the life history of many
49 invertebrates, fish, and birds, making it crucial to investigate the severity of anthropogenic impacts
50 making anthropogenic contaminants an ecological concern. Starting in 2002, the estuary underwent
51 major redevelopment and rehabilitation work (dredging to improve water circulation, reconstruction
52 of meanders, prolongation of breakwaters, and creation of an artificial island) under the Port 2000
53 project which extended the harbor and closed out the northern flank of the estuary (Tecchio et al.,
54 2016). The construction of the port fundamentally shifted hydrology and deposition of sediments
55 into the ecosystem which may have mixed effects on organisms (Dauvin et al., 2006). Sediments are
56 a sink for numerous persistent compounds and reworking sediments could potentially reintroduce
57 sequestered contaminants into the ecosystem and threaten both benthic and pelagic organisms
58 (Fisson, 2014). The project to expand the port integrated ecological considerations in an attempt to
59 mitigate anthropogenic impacts but major changes in hydrology and sedimentary characteristics of
60 the estuary caused clear modifications in benthic communities (Cuvilliez et al., 2009; Dauvin et al.,
61 2010).

62 Investigating environmental impact of such a development plan, it is functionally and
63 economically unrealistic to screen for every potential contaminant, and implementing biological tools
64 may be more cost-effective (Amiard and Amiard-Triquet, 2008). To assess the health status of the
65 Seine estuary it was proposed to use biomarkers addressing multiple levels of biological organization
66 from sub-individual endpoints to population dynamics, as biomarkers may provide early warning
67 tools to identify stress caused by contaminants (Amiard and Amiard-Triquet, 2008). The effectiveness
68 of biomarkers in biomonitoring frameworks is recognized but their deployment is limited risk
69 assessment strategies due the wide range of responses that can be observed and the effect this has
70 on interpretation of impact. Concentrations of anthropogenic contaminants can be determined by
71 chemical analysis to identify if harmful compounds are present at concentrations above the natural
72 background making it comparatively easy, when coupled with biological response, to identify
73 whether or not a site is contaminated and if amelioration efforts improve environmental conditions.

74 To assess the influence of the Port 2000 construction on the Seine estuary, the National
75 Ecotoxicology Program (PNETOX) conducted an environmental risk assessment survey during the
76 construction of the harbour (2002-2004). The project implemented a multitude of biotic and abiotic
77 endpoints and compared values to the Authie estuary. At the time of the initial study, Authie was
78 identified to be an estuary with similar environmental parameters, equivalent latitude and
79 comparatively lower levels of contamination (Durou et al., 2008). Industrialization is also absent in
80 the Authie estuary but low levels of metal and PAH contaminant are present and, in the context of
81 the study, was considered an ideal comparator for assessing the quality Seine estuary (Francescangeli
82 et al., 2020). *Hediste diversicolor* (*H. diversicolor*), a sediment-dwelling polychaete worm, was
83 selected for the study due to its status as a keystone, sentinel species with a broad geographic
84 distribution (Durou et al., 2007a; Kalman et al., 2009; Thit et al., 2015). The sampling survey

85 investigated the impact at different levels of biological organization on *H. diversicolor* (Amiard-
86 Triquet and Rainbow, 2017). The results of the project indicate that biomass and density of the
87 suprabenthos declined between 2002 and 2004 (approximately 80%) and that population levels of *H.*
88 *diversicolor* diminished too (Gillet et al., 2008; Tecchio et al., 2016). The study also highlighted
89 evidence linking anthropogenic activities in the Seine estuary to sub-individual effects in *H.*
90 *diversicolor* (Durou et al., 2008; Durou and Mouneyrac, 2007). Previous studies have demonstrated
91 that benthic communities show a steep decline after major alterations and that longer periods post-
92 construction are necessary to verify the effectiveness of remediation efforts (Meng et al., 2018). It
93 can also be unclear how to interpret data from multiple levels of biological organization in a clear and
94 concise interpretation of environmental health. When considering the response of organisms
95 however, it can be unclear how to interpret data from multiple levels of biological organization and
96 create clear conclusions on the health status of organisms in a polluted site.

97 To follow up on the longevity of adverse effects of pollution in the Seine estuary, project
98 ECOTONES (2015-2017) was initiated approximately 10 years later measuring the same biological
99 endpoints as PNETOX. Project ECOTONES aimed to assess if the overall health of *H. diversicolor* in the
100 Seine estuary improved because of remediation efforts. Interpreting organismal responses using
101 multiple levels of biological organization can often be unclear, leading to inconsistent interpretations
102 of health status in contaminated estuaries. Applying a method of biomarker response integration
103 that weights biological endpoints based on the level of biological organisation can potentially be used
104 to establish concise conclusions on health status.

105 One of the proposed methods to integrate biological responses at different levels of
106 organization is based on the use of a line of evidence (LOE) for biomarkers (Barjhoux et al., 2018; Piva
107 et al., 2011). This LOE model compiles biological responses at multiple levels of organization into an
108 integrated hazard quotient (HQ) associated with a hazard class ranking from absent (no observable
109 biological impact) to severe (significant important impact), allowing for the comparison of temporal
110 biomarker datasets through quantitative (HQ values) and qualitative (hazard classes) methods. Six
111 biological endpoints were used in the present study: oxidative stress biomarkers (catalase (Cat),
112 glutathione-s-transferase (GST), and thiobarbituric reactive substances (TBARs)), neurotoxicity-
113 specific response (acetylcholinesterase activity (AChE)), energy reserves (total lipid and glycogen
114 content) and population density. For each integrated endpoint, reference level and thresholds were
115 derived from the baseline assessment criteria (BAC) and the environmental assessment criteria (EAC)
116 previously defined (Barrick et al., 2018a, 2016) which integrate the effects of confounding factors
117 (salinity, weight of individuals and temperature) on the studied biomarkers into the analysis.

118

119 **Materials and methods**

120 *Sample site and collection*

121 The Seine estuary in northern France is a system influenced by high tides and the estuary is
122 split in three spatial areas: the Northern Flank (NF), the Central Channel (CC), and the Southern Flank
123 (SF) (Figure 1). Both PNETOX and ECOTONES sampling campaigns were carried out in the NF region of
124 the estuary. In both projects, *H. diversicolor* individuals were collected in the intertidal zone at low
125 tide in the Seine estuary (49°27'17.52" N and 0°11'19.57" E). Historical data for the spring, summer,
126 and fall seasons of the 2002-2004 period in the Seine estuary were collected from previous studies
127 (Durou et al., 2007a; Durou and Mouneyrac, 2007; Gillet et al., 2008).

128 Sampling for ECOTONES was carried out two times each year (spring and summer for 2015-2017).
129 The temperature and the salinity of water remaining on the mudflat surface were recorded using a
130 field thermometer (CG867) and a salinometer (WTW LF 196). Biological endpoints, time points and
131 sampling protocols comparable to the historical data generated in PNETOX. To analyze density, four
132 quadrats (625 cm² and 25 cm deep) were collected during each sampling campaign (Ait Alla et al.,
133 2006; Gillet et al., 2008). Quadrats were then sieved to collect organisms (200µm mesh size) and
134 organisms retained were collected and stored in 70% ethanol. For biomarker analyses, samples were
135 collected gently by hand in parallel with the collection of samples for density. Twenty sexually
136 immature worms of equivalent size (0.11±0.03g wet weight) were collected and individually analyzed
137 for enzymatic biomarkers (AChE, GST, Cat, and TBARs) and twenty worms of equivalent size were
138 collected and individually analyzed for energetic reserves (glycogen and lipids). Samples were
139 immediately frozen in liquid nitrogen, transported to the laboratory, and stored at -80°C until
140 analysis.

141

142 *Biomarker analysis*

143 Biomarkers in the present study were analyzed following protocols used in the PNETOX
144 program. Briefly, enzymatic biomarker responses were assessed using the techniques described in
145 Durou et al., 2007. Samples were individually homogenized in buffer solution (0.08 M Tris, 2 M NaCl,
146 1 mM of 1,4-Dithiothreitol, and cocktail protease inhibitor diluted to 1:1000 at pH 7.4 and 4 °C) in a
147 1:3 ratio of tissue to the solution using a mortar and pestle. The homogenates were then centrifuged
148 for 30 minutes at 9000×g. Total protein content was calculated by following the Bradford assays
149 (Bradford, 1976; Galgani and Bocquene, 1991). GST activity was determined spectroscopically at
150 340nm by measuring the rate that 1-choloro-dinitrobenzene is conjugated with reduced glutathione
151 (Habig et al., 1974). Cat activity was calculated by measuring the consumption of H₂O₂
152 spectroscopically at 240 nm. Results were expressed as µmoles.min⁻¹.mg of protein⁻¹ (Aebi, 1984).
153 AChE activity was calculated by using a coupled reaction measuring the catalyzation of
154 acetylthiocholine to produce thiocholine (which is then reduced by 5,5'-Dithiobis(2-nitrobenzoic acid)
155 to produce thionitrobenzoate (Bocquené et al., 1990). The rate of the reaction was measured by
156 colorimetric spectroscopy. Lipid peroxidation was measured by the formation of thiobarbituric
157 reactive substances quantified by malondialdehyde absorbance at 530 nm (Knight et al., 1988).

158 Energetic reserves were analyzed following the protocol previously described in Durou et al.
159 (2007). Individuals were ground into a powder using liquid nitrogen and then prepared as a
160 homogenate in a citrate buffer (0.1 M, pH 5.0). Total lipid content was determined following the
161 method defined in (Frings et al., 1972). Glycogen content was determined by partitioning the sample
162 in two (with or without enzymatic digestion by amyloglucosidase) following the protocol described in
163 (Carr and Neff, 1984a).

164

165 *Data integration within Biomarker index and development of LOE model*

166 To establish a baseline that accounted for natural variation, the method described in Barrick
167 et al. (2016) was applied. Multi-polynomial regression models were previously developed for each
168 biomarker to integrate confounding factors (salinity, temperature, and weight of individual) into the
169 analysis, establishing thresholds of expected natural variation using data from the Authie estuary as a
170 reference point. Authie was used as the reference point for this study to maintain comparison with
171 the historical data and, while the use of several reference sites is ideal, there is limited comparable

172 data available for other estuaries. For each regression model, a baseline assessment criterion (BAC)
173 was used to define the expected range of variation that would occur naturally in healthy individuals.
174 A banding approach was used to define the threshold of disturbance, also called environmental
175 assessment criterion (EAC), with twice the lower limit of the BAC used for stress-induced inhibition
176 endpoints (AChE, energy reserve assays, and density) to indicate the biomarker is severely impacted.
177 Formulas were previously defined for biomarkers (AChE, GST, CAT, TBARS, glycogen, and lipids) in
178 Barrick et al. (2016, 2018). For population density, a regression model was calculated following the
179 same method. BAC/EAC values were established for each sampling periods in the Seine estuary using
180 confounding factors measured during sampling periods for both projects.

181 To characterize *in situ* biological responses for *H. diversicolor* for integrated biomarker
182 responses an LOE model was generated following a previously defined method with some
183 adjustments to fit data collected (Barjhoux *et al.*, 2018, Piva et al., 2011). The application of the LOE
184 model requires the establishment of reference values (or control values), thresholds (Th) values
185 indicating the range of expected natural variation, and an effect threshold above which physiological
186 disturbances are expected to occur. Mean values from the BAC models were used as reference
187 values and the Th value was calculated from the lower or upper limits of the BAC depending on how
188 the biological responses change under stress conditions, i.e. the 'ecologically relevant' direction of
189 biomarker modulation. Effect threshold values were derived from EAC values for each biological
190 endpoint. In the LOE model, only unilateral modulations were taken into consideration. Details of the
191 LOE parametrization and calculations are presented in the supplemental material and summarized
192 below.

193 To further develop the model, measured responses were expressed as a percentage change
194 over the respective reference value and divided by the Th value. A correction factor was applied to
195 the resulting ratio using the following method: a 0.2-factor for values less than or equal to the Th
196 values, a 0.5-factor for responses above the Th value but lower than the effect threshold, and a 1-
197 factor for values that exceeded the effect threshold. These calculations resulted in an 'effect' value
198 E(i) for each biological response which was reported on an evaluation grid for hazard classification
199 ranging from absent to severe. E(i) values were then weighted (Ew(i)) according to the biological
200 significance of the considered biomarker following the approach described in Piva et al. (2011): a
201 weight of 0.75 was applied to TBARS and AChE, a 0.5-weight was assigned to CAT and GST, a 1.2
202 weight was given to lipid and glycogen, and a weight of 2.8 for density measurements as an
203 intermediate value between mortality (3.0), histopathology and reproductive failure (2.4).

204 Finally, a cumulative biomarker hazard quotient (BiomHQ) was generated by calculating the
205 weighted average of the Ew(i) values of biomarkers attributed to the moderate class of hazard and
206 summing the Ew(i) values with responses belonging to the major or the severe class of hazard. The
207 contribution of each endpoint and class of endpoint to the BiomHQ value was also calculated as
208 described by Barjhoux et al. (2018). Another global index (BiomHQ index) was also generated by
209 adding the percentage of biomarkers divided into the different classes of hazard weighed with
210 coefficients increasing with the attributed hazard (from 0.7 for absent to 8 for severe; according to
211 Piva et al. 2011). This new index was reported on an evaluation grid for a global hazard classification
212 (absent to severe).

213

214 **Results**

215 When assessing the health status of *H. diversicolor* using individual biomarker hazard
216 rankings many of the biological endpoints demonstrated wide variations ranging from "absent" to

217 “severe” between sampling campaigns (Table 1). Of the endpoints used in the present study TBARS,
218 glycogen and density were the endpoints the indicated stress. Annually, hazard rankings for the
219 PNETOX campaign suggested stress response for TBARS, AChE, GST, catalase, lipids, glycogen and
220 density for 2002 whereas in 2003 AChE, GST, glycogen and density indicated stress and in 2004
221 only GST and density demonstrated a stress response. For project ECOTONES TBARS, AChE, lipids,
222 glycogen and density consistently suggested a stress response for 2015-2017 with catalase indicating
223 moderate stress in 2015.

224 Individual biological endpoints were then weighted following the approach used in Piva et al.
225 (2011) and results were integrated into biomarker hazard quotients (BiomHQ) to classify stress
226 response (Figure 2). This index represents the cumulative response profiles of all biological endpoints
227 relative to reference values and can be interpreted as a quantitative indicator of the intensity of
228 deviation from expected values (ie out of the range of basal variations defined by the reference and
229 the threshold T_h values). Results from the PNETOX program showed limited variations of the
230 BiomHQ values, with maximal values (13-15.5) observed between July 2002 and February 2003 and
231 the lowest (0-3) between November 2003 and May 2004. On the contrary, BiomHQ values calculated
232 with ECOTONES results proved to be generally higher and more variable with indices reaching about
233 20 and 35 in March 2015 and July 2015, respectively. Individual BiomHQ values are calculated based
234 on the number of biomarker responses/ severity of response for each data set. In some cases this
235 leads to lower values indicating higher stress response (e.g. 2002 vs 2003). The BiomHQ index, a
236 more qualitative description of the biological disturbance observed at each sampling time, was
237 calculated according to the weighted distribution of biomarkers within the different classes of risk
238 attributed for each $E(i)$ value. This second index is used to attribute the risk classification for each
239 sampling period and the annual averages (see figure S1 in the supplemental material).

240 The contribution of each biomarker to the interpretation of hazard were also assessed
241 (Figure 3). In many of the sampling campaigns for PNETOX, oxidative stress (mainly TBARS and GST),
242 neurotoxicity (AChE), and populational (density) markers were major contributing factors to the
243 BiomHQ values. Other responses such as energetic reserves and CAT also influenced the level of
244 hazard in some of the PNETOX sampling campaigns but more punctually. For the ECOTONES sampling
245 campaign, TBARS were a major contributor in defining the health status for *H. diversicolor* the Seine
246 estuary. Energetic reserves contributed more to the hazard classification in the ECOTONES sampling
247 campaign compared to the PNETOX samples. AChE, CAT, and population density also factored into
248 the level of environmental hazard in ECOTONES but their contribution was dependent on the sample
249 period and no clear profile could be identified.

250 Annually, the population density was a major indicator for 2003 and 2004 whereas TBARS,
251 neurotoxicity, and energetic reserves (in particular glycogen) were the most contributive biological
252 endpoints for 2002 and 2015-2017. As observed for the first PNETOX campaigns, the highest
253 BiomHQ values are associated with more complex contribution profiles indicating that impact can be
254 detected at different levels of response.

255

256 **Discussion**

257 Polychaete worms are ideal target organisms for monitoring ecosystem health as they are
258 sensitive to anthropogenic activity and play a crucial role as contaminant vectors across the trophic
259 chain (Green Etxabe et al., 2021). The objective of the present study was to investigate if remediation
260 efforts in the Seine estuary had improved the health status of *H. diversicolor* in a way that accounts
261 for natural variability using a multi-biomarker approach following an integrative method of analysis.

262 The BAC/EAC model attempts to account for effects environmental parameters (temperature, salinity
263 and weight) on the biological endpoints used in the study by defining the range of values expected to
264 naturally occur for each endpoint. The results of the model demonstrated large fluctuations
265 seasonally and annually for all biological endpoints, suggesting that natural variation may not be
266 completely accounted for and that longer time series may be necessary to characterize this
267 relationship more appropriately. Additional research is necessary to further clarify the complex
268 relationship between biological endpoints, natural variation and effects of anthropogenic activity
269 (Galloway, 2006). Measurement of temperature and salinity in the ECOTONES project were
270 measured to conform to the how data was collected in the PNETOX campaign and measured
271 environmental temperature and salinity at the time of sampling. Using an average temperature and
272 salinity across several timepoints might improve the model's ability to predict how these parameters
273 influence measured biological endpoints. Integrating additional parameters such as granulometry,
274 available organic matter and reproductive status can also significantly improve the model as these
275 endpoints can alter metabolic activity which, in turn, can influence subindividual endpoints (Díaz-
276 Jaramillo et al., 2011).

277 Annually PNETOX data indicated less stress in 2003 and 2004 when compared to the initial
278 sampling in 2002 which would be consistent with recovery after a major environmental perturbation
279 like the construction of the port. Data for the ECOTONES campaign was relatively consistent 2015-
280 2017 which suggested the site was majorly impacted. This observation would indicate that despite
281 the 10-year difference between the two projects, *H. diversicolor* populations in the Seine estuary
282 were still majorly impacted and effects of contamination likely persistent and chronic (please see
283 supplemental materials for data regarding measured contaminants present. The site used to collect
284 *H. diversicolor* in the Seine estuary is an active shipping zone and has been demonstrated to have
285 high concentrations of PAHs, PCBs and organochlorine pesticides which have been previously, and
286 proportionally, accumulated in *H. diversicolor* (Durou et al., 2007a; Lesueur et al., 2015). The Seine
287 estuary also has high concentrations of metal in the sediment with previous studies indicating a poor
288 correlation between metal concentrations in the environment and concentrations in *H. diversicolor*
289 (Amiard-Triquet et al., 2007; Berthet et al., 2015; Saiz-Salinas and Francés-Zubillaga, 1997).
290 Nonetheless, chronic exposure of *H. diversicolor* to both persistent organic pollutants and metallic
291 trace elements present in the sediment of the Seine estuary may be responsible for the effects
292 highlighted by the LOE model.

293 Biological endpoints used in the study account for a wide range of responses including
294 neurotoxicity, oxidative stress, energetic reserves, and population density. Surprisingly, endpoints
295 that consistently suggested adverse effects were mainly TBARS, glycogen and density suggesting that
296 *H. diversicolor* is impacted at multiple levels of biological organization. Results also indicated that, of
297 the subcellular endpoints, TBARS was one of the more consistent biomarkers. One potential
298 explanation for this observation is that TBARS are indicative of damage and suggest that defense
299 mechanisms are consistently overwhelmed in the Seine estuary, implying chronic effects. Enzymatic
300 biomarkers (AChE, GST and Cat) displayed seasonal patterns which is unsurprising as the previous
301 BAC/EAC models indicated a wide range of natural variation (Barrick et al., 2018b; Beliaeff and
302 Bocquené, 2004; Hagger et al., 2006). It has also been demonstrated that enzymatic biomarkers can
303 be influenced seasonal variation in solar exposure and adjustments to energetic budgets (Díaz-
304 Jaramillo et al., 2011). Future adjustments may need to consider a piece-wise integrative model in
305 order to investigate the relationship between levels of biological organization at specific time points,
306 in particular the relationship between subcellular responses and dynamic energy budgets .

307 When considering the results of energetic reserves, the LOE model suggested glycogen levels
308 are impacted in the Seine estuary, which is consistent with the BAC model previously generated
309 (Barrick et al., 2016). Glycogen also had a considerable contribution to the interpretation of
310 environmental health status suggesting that it may be a useful endpoint in identifying sites adversely
311 impacted by anthropogenic activity. With respect to lipids there was some evidence suggesting
312 moderate stress but overall was less impacted than glycogen. This may suggest that glycogen is more
313 responsive to environmental condition which may make it an ideal endpoint for measuring
314 anthropogenic activity. One potential explanation for this is that glycogen is the primary energy
315 resource (maintenance, reproduction, growth) when accounting for environmental stress (Carr and
316 Neff, 1984b). It is also important to consider that energetic reserves are highly dependent on the
317 reproductive status of the organisms and that contamination may alter biological clocks (Pook et al.,
318 2009). Previous work on the PNETOX data demonstrated that the sexual reproductive status of *H.*
319 *diversicolor* in the Seine were equivalent to individuals in Authie, suggesting an allocation of energy
320 towards reproduction rather than growth (Durou et al., 2007b). Integration of reproductive status
321 into the model would be beneficial as it would help characterize the effects of this relationship on
322 other biological endpoints.

323 Population density values consistently fell outside the range of expected natural variation,
324 providing evidence that the Seine is still majorly impacted and potentially influencing the ecosystem.
325 This is consistent with a previous interpretation which suggested that low population densities may
326 be due to irrigation and sediment reworks in the Seine estuary (Gillet et al., 2008). It is however
327 important to note that polychaete population densities can display significant variations across time
328 and space (Kristensen, 1984). Despite Authie being considered a good comparator estuary for the
329 Seine intersite differences, such as nuances in sediment texture and algal cover, can potentially make
330 it an unreliable comparator site for density assessments (Alzeny et al., 2021). Integrating multiple
331 references sites and accounting for these environmental parameters can potential improve the
332 quality of the model. Perturbations to the density of *H. diversicolor* indicated impact during the
333 PNETOX sampling campaign may be attributed to immediate alterations to the sediment dynamics as
334 result of the construction process rather than the presence of contamination. The results of the
335 model suggested 'moderate' to 'major' effects towards *H. diversicolor* density across the 10-year
336 period, suggesting the site is still impacted. Previous research on macrofaunal composition in the
337 Seine estuary are resilient to anthropogenic perturbation and one potential explanation for the
338 interpretation of the model in the present study is that the response profile is localized (Bacouillard
339 et al., 2020). Future alterations to the model should consider including multiple sampling sites in the
340 Seine estuary.

341 No true reference values exist but the model attempts to account for expected environmental
342 variation through the application of a BAC/EAC index. While this approach allows for clear, concise
343 results for communicating environmental health to regulatory bodies, it is important to note that
344 additional research is necessary to clarify the complex relationship between biological endpoints,
345 natural environmental variation and effects of anthropogenic activity (Galloway, 2006). Some of the
346 endpoints used in the model demonstrated clear seasonal patterns indicating that longer time series
347 may be necessary. It also necessary to look at additional endpoints linked to seasonal variation such
348 as nutrient load, turbidity, and sediment transport.

349 The LOE model establishes a first step in developing a comprehensive model to provide an holistic
350 assessment of *H. diversicolor* to improve the assessment of health status. Improvements can be
351 made to the initial model by including other biological endpoints such as genotoxicity, reproductive
352 success, or scope for growth. This was not done in the present study due to a lack of necessary data

353 to develop relevant BAC indices for these endpoints. In addition to this, different biological endpoints
354 have varying degrees of sensitivity to environmental disturbance and it may be necessary to clarify
355 these relationships in future studies. Through the integration of population density into the LOE
356 model it becomes possible to extrapolate how sublethal endpoints can lead to adverse effects on the
357 population. Future research will need to expand on the relationship between endpoints as well as
358 how environmental parameters may influence these interpretations.

359

360 **Conclusion**

361 “The proposed model provides a holistic assessment of the status of *H. diversicolor* individuals in
362 the Seine estuary but to be effective there needs to be effective integration with other LOEs, in
363 particular sediment type and concentrations of contaminants, to effectively establish a weight of
364 evidence approach that characterizes environmental quality. The present study proposes a first
365 attempt at developing an LOE model for *H. diversicolor* that integrates subcellular endpoints,
366 subindividual and populational effects to establish a holistic approach to environmental health. This
367 approach was applied to biological responses analyzed in the sentinel species *H. diversicolor*
368 following two long term studies in the Seine estuary. The LOE model implemented the use of a
369 BAC/EAC model which attempts to correct for variation due to natural variations such as
370 temperature, salinity and weight of the organisms. The quality of this assessment can be improved
371 upon by integrating time series data for the environmental parameters, which could potentially
372 capture how climate change influences *H. diversicolor*.

373 There are however major limitations with the results as the data demonstrates large seasonal
374 variations in the interpretation of health status for the biomarkers and future research will need to
375 investigate how to account for this. The use of only one reference site to establish the BAC/EAC index
376 also limits the efficacy of the model and endeavors should be made establish additional data at other
377 low impact sites. Finally, the interpretation of environmental impact is also hampered by the lack of
378 of contaminant data and the results would be strengthened by chemistry data.

379 Overall, the present study demonstrates that *H. diversicolor* could be an effective model
380 organisms for environmental hazard assessment. The model could however be improved by
381 additional endpoints, such as genotoxicity and reproductive stage, can further describe the health of
382 individuals in the Seine estuary. Results suggested that *H. diversicolor* in the Seine estuary are
383 moderately to majorly impacted by environmental condition, indicating that the status of the Seine
384 estuary has not notably improved between the two sampling campaigns. This however is only one
385 line of evidence and other lines of evidence need to generate in order to effectively characterize the
386 impact human activity has had on the Seine estuary, in particular coupling additional
387 physicochemical parameters such as contamination levels in the environment and bioaccumulation
388 measurements in key species into a weight of evidence approach.”

389

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394

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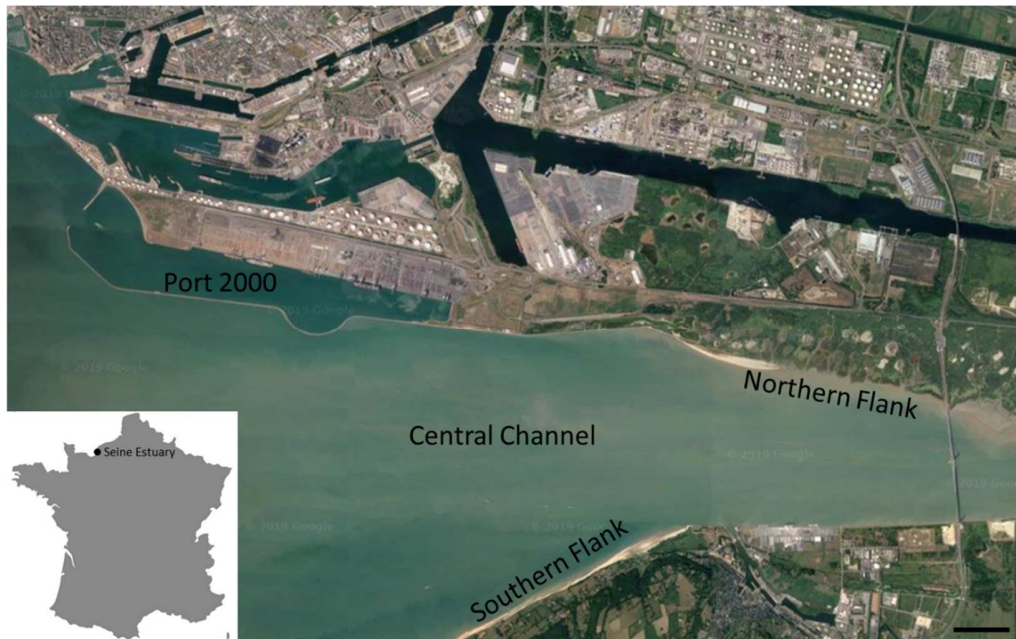


Figure 1: Location of the study area in the Seine estuary – Northern flank. Black bar indicates 1km. (Credits: Google Maps, Images @2019 Google, Data SIO, NOAA, U.S. Navyn NGA, GEBCO, Images @2019 CNES, Airbus, Landsat, Copernicus, Maxar Technologies, Données cartographique @2019).

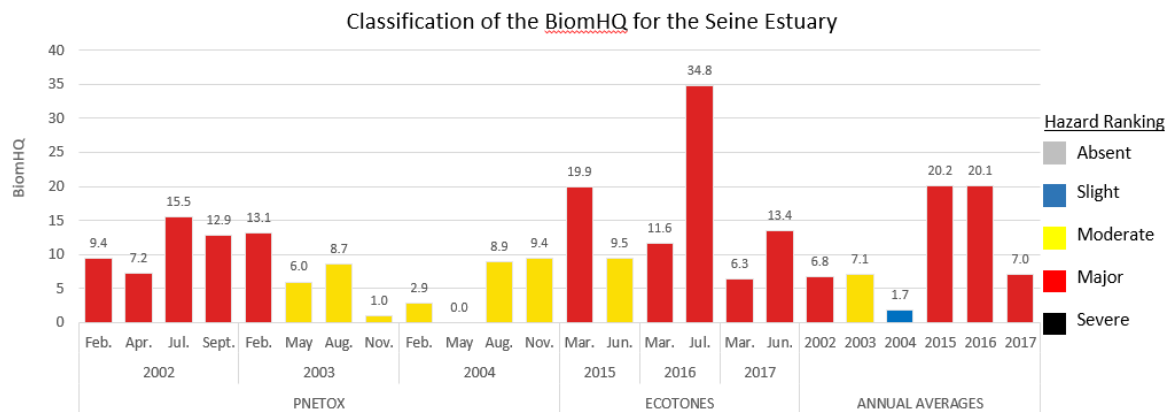


Figure 2 : Evaluation and classification of an integrated assessment of biological endpoints measured in the Seine estuary. Values are based on the biomarker hazard quotient (BiomHQ) and indicate the degree in which biological endpoints deviated from the range of expected natural variation.

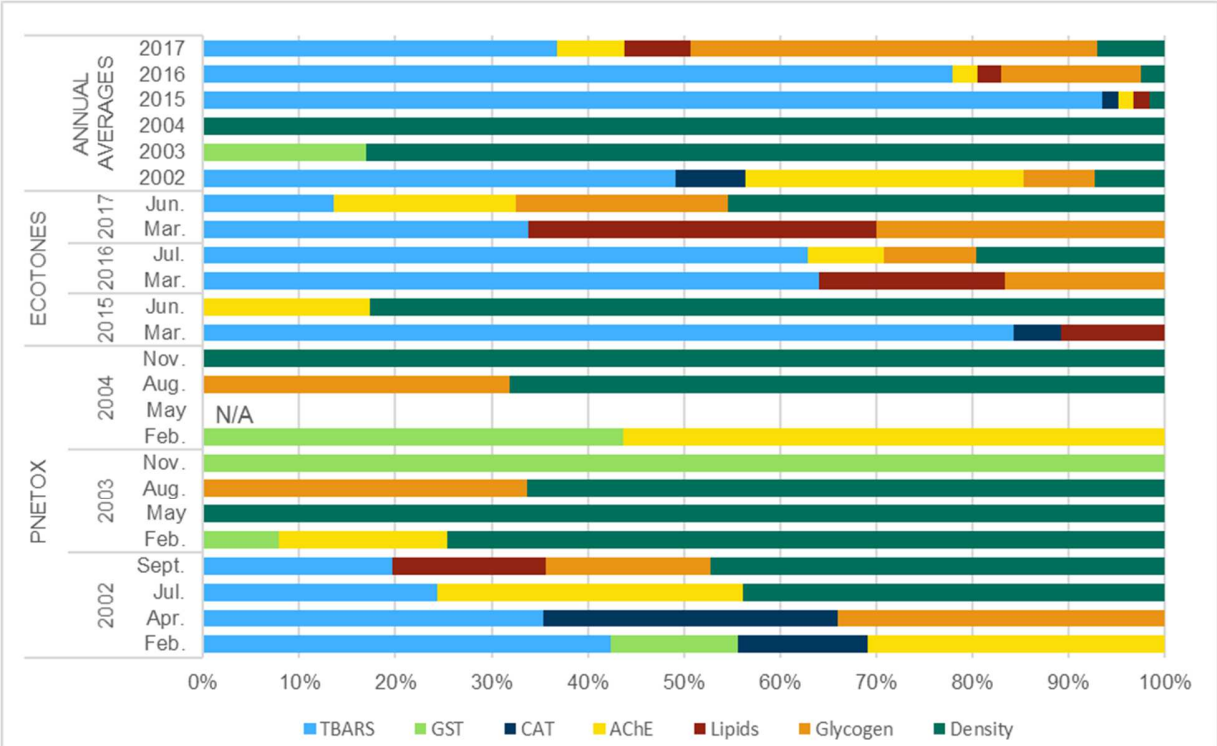


Figure 3: Contribution of each biomarker to the BiomHQ value calculated for each sampling campaign. N/A indicates that contributions could not be calculated due to BiomHQ value equals to 0.

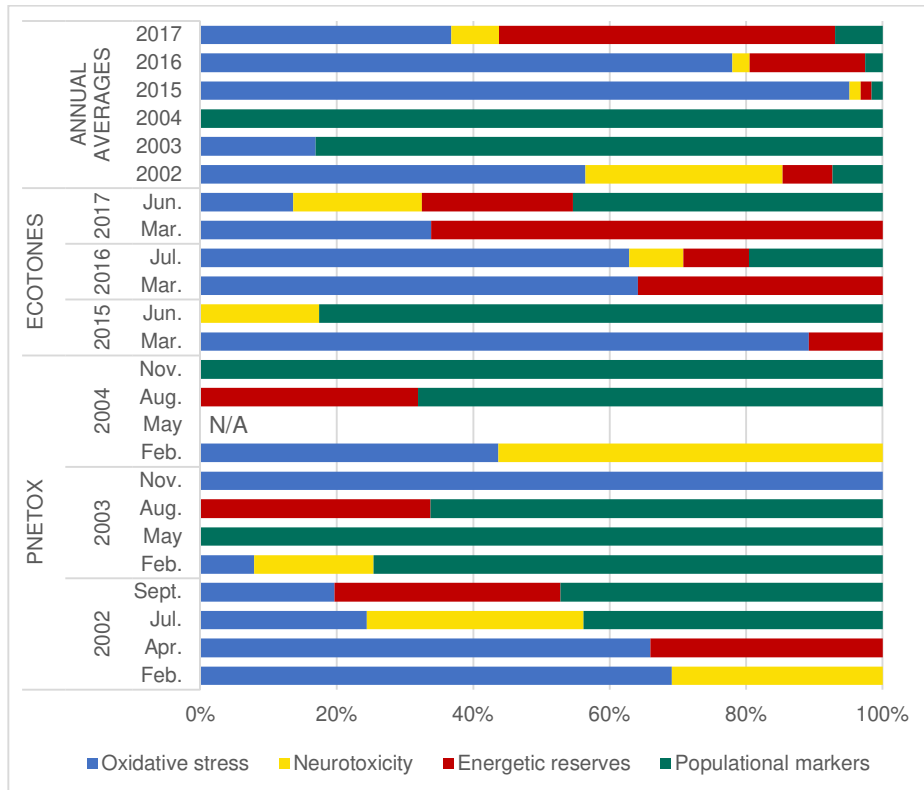


Figure 4: Contribution of each class of biological endpoints to the BiomHQ value calculated for each sampling campaign. N/A indicates that contributions could not be calculated due to BiomHQ value equals to 0.

Table 1: Classification of hazard for biological endpoints within the Biomarker LOE model, based on the calculated effect (E(i)) values. Results are presented based upon each sampling campaign and annually.

Program	Year	Month	TBARS	AChE	GST	Catalase	Lipids	Glycogen	Density
PNETOX	2002	Feb.	Severe	Severe	Major	Major	Slight	Absent	Slight
		Apr.	Severe	Absent	Absent	Severe	Slight	Major	Absent
		Jul.	Severe	Severe	Absent	Absent	Absent	Slight	Major
		Sept.	Severe	Absent	Absent	Absent	Major	Major	Major
	2003	Feb.	Absent	Severe	Major	Absent	Slight	Absent	Severe
		May	Absent	Absent	Absent	Absent	Absent	Absent	Major
		Aug.	Slight	Absent	Absent	Absent	Absent	Major	Major
		Nov.	Absent	Absent	Major	Slight	Absent	Absent	Slight
	2004	Feb.	Absent	Major	Major	Absent	Slight	Absent	Absent
		May	Absent	Absent	Absent	Absent	Absent	Absent	Slight
		Aug.	Absent	Absent	Absent	Absent	Absent	Major	Major
		Nov.	Absent	Absent	Absent	Absent	Absent	Absent	Severe
ECOTONES	2015	Mar.	Severe	Absent	Absent	Major	Major	Slight	Absent
		Jun.	N/A	Major	Absent	Absent	Absent	Absent	Major
	2016	Mar.	Severe	Absent	Absent	Absent	Major	Major	Absent
		Jul.	Severe	Severe	Absent	Absent	Absent	Major	Major
	2017	Mar.	Severe	Absent	Absent	Absent	Major	Major	Absent
Jun.		Major	Severe	Absent	Absent	Absent	Major	Major	
ANNUAL AVERAGES	2002		Severe	Major	Absent	Moderate	Slight	Moderate	Moderate
	2003		Absent	Slight	Moderate	Absent	Absent	Slight	Major
	2004		Absent	Absent	Slight	Absent	Absent	Absent	Moderate
	2015		Severe	Moderate	Absent	Moderate	Moderate	Slight	Moderate
	2016		Severe	Moderate	Absent	Absent	Moderate	Major	Moderate
	2017		Severe	Moderate	Absent	Absent	Moderate	Major	Moderate