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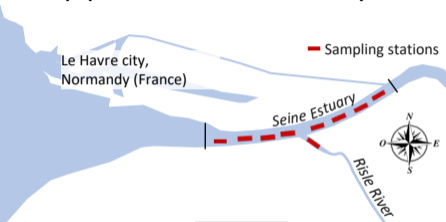
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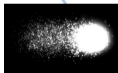
# Sperm DNA integrity of *Palaemon longirostris* population from the Seine estuary



*Palaemon longirostris*



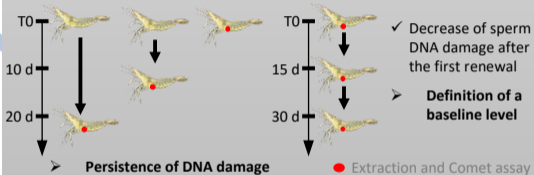
Sperm cell



Comet assay

## 1) Baseline level in laboratory experiments

- **Passive recovery** (*i.e.* transfer in healthy conditions)
- **Active recovery** (*i.e.* transfer in healthy conditions + forced renewal of spermatophore)



## 2) Deployment in biomonitoring

- 6 sampling campaigns
  - ✓ Validation of the developed approach
  - **Abnormal sperm DNA damage on all stations during the six campaigns**

1 **Assessment of sperm DNA integrity within the**  
2 ***Palaemon longirostris* (H. Milne-Edwards, 1837)**  
3 **population of the Seine estuary**

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13 **Abstract**

14 The interpretation of biomarkers *in natura* should be based on a referential of expected values in uncontaminated conditions. Nevertheless, to  
15 build a reference data set of biomarker responses in estuarine areas, which receive chronic pollution loads due to their transition position  
16 between continent and sea, is impossible. In this context, the aim of the present work was to propose the use of laboratory recovery period to  
17 define a baseline for the measurement of sperm DNA damage by Comet assay in the estuarine prawn *Palaemon longirostris*. For that, sperm  
18 DNA integrity was observed after both a passive (*i.e.* 20 days in a clean environment) and an active (*i.e.* forced renewal of spermatophores)  
19 recovery of wild *P. longirostris* specimens from the Seine estuary, in laboratory conditions. Then, the levels of sperm DNA damage recorded  
20 within the *P. longirostris* population of the Seine estuary, during six campaigns of sampling from April 2015 to October 2017, have been  
21 interpreted according to the defined threshold values. The results showed a persistence in the level of DNA damage after 20-day in clean  
22 environment with the passive recovery. This strategy was inconclusive to reach a baseline level but it revealed the lack of DNA repair  
23 mechanisms. For the active recovery, a decrease of 54 % of the level of DNA damage has been observed after the first renewal of  
24 spermatophores and this level stabilized after the second renewal. On the basis of this second strategy, we defined a mean basal value of  
25 sperm DNA damage of 54.9 A.U. and a maximum threshold of 69.7 A.U. (*i.e.* 95 %-CI). The analysis of the results using the reference value  
26 highlighted significant abnormal sperm DNA damage within the native population of *P. longirostris* from the Seine estuary on all stations  
27 during the six-sampling campaigns.

28

29 **Key words: Comet assay, Biomonitoring, Baseline, Laboratory recovery, Crustaceans.**

30

31

## 32 1. Introduction

33 Estuaries are among the most productive ecosystems supporting a wide variety of species, many of which  
34 present a potential commercial interest (Menezes et al., 2006). These systems have a predominant role in many  
35 aspects of living organisms such as nursery, food zone and migratory route (Chapman and Wang, 2001).  
36 Nevertheless, due to their transitional position between continent and sea, the majority of estuaries are submitted  
37 to continual inputs of a large diversity of contaminants. Consequently, the assessment of transitional waterbodies  
38 quality and health status is an important issue which requires the development of operational biomonitoring  
39 methodologies.

40 Biomarkers are considered as relevant biomonitoring tools to establish causal relationships between the exposure  
41 to chemicals and the impacts on organisms, integrating the aspects of bioavailability and synergetic effect of  
42 chemical mixture (Hanson et al., 2010). They are starting to be considered in the regulatory framework of  
43 environmental surveys in Europe, as for example in the UE Marine Strategy Framework Directive (2008/56/EC).  
44 For many years, the major part of the *in situ* applications of biomarkers was based on the upstream/downstream  
45 approach involving the comparison between a reference and an impacted site (Flammarion and Garric, 1997;  
46 Flammarion et al., 2002). This approach has been well established and showed its relevance to assess the quality  
47 of sites presenting similar physicochemical parameters. However, estuarine ecosystems are subject to significant  
48 changes in physicochemical conditions at different spatial (*e.g.* upstream-downstream salinity gradient) and  
49 temporal scales (*e.g.* tidal flow) (Lobry et al., 2006), making the upstream/downstream comparisons unsuitable.  
50 More recently, the construction of a chronological and/or spatial reference within un-impacted population,  
51 integrating the natural variability as incertitude source (*i.e.* both intrinsic biotic and environmental factors), was  
52 proposed as a relevant strategy to establish baselines and threshold values for biomarker responses (Barrick et  
53 al., 2016; Hagger et al., 2008; Hanson et al., 2010; Jubeaux et al., 2012; Lacaze et al., 2011a; Xuereb et al.,  
54 2009). This approach allows the deployment and the interpretation of biomarkers on a large spatial scale, without  
55 having to refer in parallel to reference stations. If such levels of finalization have been achieved for some  
56 biomarkers in some marine and freshwater species (*e.g.* Coulaud et al., 2011; Erraud et al. 2018; Lacaze et al.,  
57 2011a; ICES, 2011; Xuereb et al. 2009), they are particularly difficult to reach in estuarine species. This  
58 statement is due in large extent to the difficulties to characterize a reference station in open systems submitted to  
59 the pollutant discharges in the up-stream part of their watersheds. On the other hand, direct comparisons between  
60 native populations of exclusively estuarine species from different estuaries must be done with care the influence  
61 of different adaptive mechanisms (Rank et al., 2007).

62 It is however a great concern to deal with such difficulties in order to propose methodologies allowing to assess  
63 the health of organisms inhabiting these important ecosystems. An alternative strategy based on the restoration  
64 of a basal response after a recovery phase in laboratory healthy conditions has been demonstrated as suitable to  
65 determine references for some biomarkers (*e.g.* EROD, DNA integrity of somatic cells) in the black sea bream,  
66 *Acanthopagrus butcheri* (Webb and Gagnon, 2013). The relevance of this approach should be investigated for  
67 other biomarker/species combinations.

68 The Seine estuary (France) is considered as one of the most polluted estuaries in Europe, subject to an important  
69 anthropogenic pressure due to its location at the outlet of the Seine watershed (Burgeot et al., 2017; Cachot et al.,  
70 2006; Carpentier et al., 2002; Meybeck et al., 2004). The Seine basin represents a territory of 78 600 km<sup>2</sup> and  
71 welcomes more than a quarter of the French population (Dévier et al., 2013). In addition to the Parisian  
72 metropole, the Seine estuary receives anthropogenic pressure from two major nearshore agglomerations and  
73 major harbours (*i.e.* Rouen and Le Havre); four major industrial areas (*i.e.* Elbeuf, Rouen, Port Jérôme and Le  
74 Havre); and many agricultural areas bordering the Seine River. The perpetual chemical overflow of contaminants  
75 of the Seine River, added to the remobilisation of contaminants linked with the hydro-sedimentary function, the  
76 maritime and fluvial traffic which requires a daily dredging, the industrial past and the important intake of the  
77 upstream watershed of this estuary together make this estuary largely affected by a wide range of contaminants  
78 (Cailleaud et al., 2007a, b). This estuary is therefore particularly prone to genotoxic pressure since the Seine  
79 River receives inputs of a large variety of micro-pollutants such as polycyclic aromatic hydrocarbons (PAH)  
80 (Motelay-Massei et al. 2007), trace metals (Grosbois et al. 2006) and polychlorinated biphenyls (PCB) (Meybeck  
81 et al. 2004).

82 Genotoxic biomarkers are considered as integrative tools, able to provide complementary informations to  
83 chemical and ecological analyses in field monitoring (Lacaze et al., 2011a). Indeed, the study of genotoxicity  
84 represents a major challenge for the environment preservation due to the wide range of genotoxic substances (*i.e.*  
85 more than a third of the anthropogenic compounds released into the aquatic environment), and their  
86 environmental impact and the possible ecological consequences, particularly on the survival and the renewal of  
87 certain wild populations (Claxton et al., 1998; Ohe et al., 2004). For these reasons, genotoxic assessment in the  
88 Seine estuary has started in the 2000s and has been the focus of Research until now. In spite of the diversity of  
89 genotoxic compounds, only PAHs have been extensively investigated in the Seine estuary, and majority of works  
90 were based on bioassays assessing the genotoxicity potential of sediments or suspended particulate matter  
91 using bacterial strain, cell lines (*i.e.* SOS Chromotest and Ames test) or exotic species (Barjhoux et al., 2012;

92 Vicquelin et al., 2011; Vincent-Hubert et al., 2017). Up until now, only few studies measured genotoxic effects  
93 in somatic cells of indigenous organisms living in the Seine estuary such as the flounder *Platichthys flesus*  
94 (Marchand et al., 2004) and the mussels *Dreissena polymorpha* for the upstream part and *Mytilus edulis* for the  
95 mouth part of the estuary (Rocher et al. 2006, Le Goff et al. 2006). Moreover, no crustacean group has been  
96 investigated in the Seine River in spite of its ecological and ecotoxicological interest.

97 Among crustaceans, *Palaemon longirostris* (Milne Edwards, 1937) is a typical estuarine species that completes  
98 its whole life cycle in brackish water (González-Ortegón et al., 2006), which is present from the north of Africa  
99 up to Western Germany and North-East England (view in Béguer et al., 2009). *Palaemon longirostris* is the most  
100 common and abundant prawn in the Seine estuary, as in other large estuaries of the Atlantic French coast.

101 In this context, the global aim of our study consists in the development of Comet assay on Palaemonid prawns  
102 spermatozoa to assess the potentiality of the sperm DNA integrity to be proposed as a relevant biomarker for the  
103 surveys of European coastal and estuarine water bodies. Our investigation focuses on spermatozoa since they are  
104 in most cases considered to be sensitive to contamination due to their inability to prevent oxidative stress and to  
105 repair DNA damage (Aitken et al., 2004). In addition, assessment of genotoxicity on this cellular type is of  
106 obvious interest regarding its key role in reproduction success (Lacaze et al., 2011a; Lewis and Galloway, 2010;  
107 Devaux et al. 2011, 2015; Santos et al., 2013a,b). During previous studies, the methodological procedure of  
108 Comet assay was adapted and optimized for *Palaemon* sp spermatozoa and a reference data distribution was  
109 defined for the coastal species *P. serratus* (Erraud et al., 2018 a,b). The objective of this present work was to  
110 propose an alternative strategy to define a baseline for the native population of *P. longirostris* living in the Seine  
111 estuary, in order to assure a robust interpretation of this marker in this complex ecosystem. In invertebrate  
112 species, little information is available concerning DNA repair of spermatozoa and in a lesser extent in  
113 Palaemonidae spermatozoa in spite of their particularities, which differ from those of the aquatic invertebrates in  
114 both form and function (Braga et al. 2013). So, in the first step, different passive and active recovery approaches  
115 were performed on wild specimens (*i.e.* naturally exposed to Seine contamination) transferred to healthy  
116 conditions in laboratory to try to define the basal level of sperm DNA damage. In the second step, the sperm  
117 DNA integrity of the Seine estuary *P. longirostris* population was assessed during 6 sampling campaigns, in  
118 2015, 2016 and 2017.

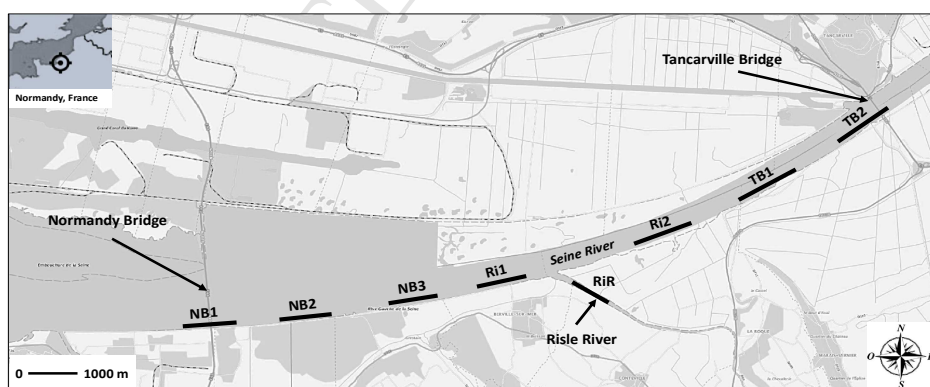
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## 120 2. Materials and methods

## 121 2.1. Field collection and maintenance in laboratory conditions

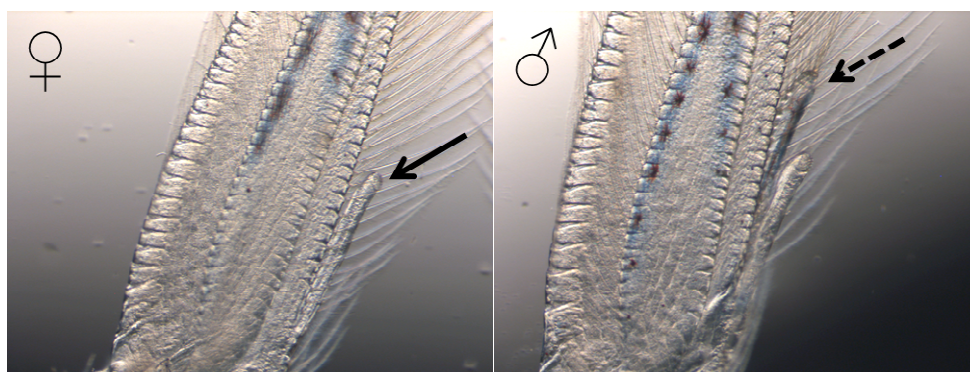
122 Adult specimens of *Palaemon longirostris* (i.e.  $32,4 \pm 3,6$  mm of total body size) were collected along the  
 123 salinity gradient in the mesohaline zone of the Seine estuary, between the Normandy and the Tancarville  
 124 Bridges, in April 2015, January 2016, May 2016, October 2016, April 2017 and October 2017 (Normandy,  
 125 France; Fig 1). Eight stations were monitored in each sampling campaign in order to find males, considering the  
 126 movement of the salinity gradient at the different seasons (Fig. 1): three stations in the vicinity of the Normandy  
 127 bridge (i.e. NB1 = Normandy bridge; NB2 = Grestain abbey; NB3 = Grestain); two stations at the mouth of the  
 128 Risle River (i.e. Ri1 = Berville-sur-mer ; Ri2 = Blanc banc); one station in the Risle River (i.e. RiR); two stations  
 129 at the proximity of the Tancarville bridge (i.e. TB1 = Pointe de la Roque; TB2 = Tancarville bridge).

130 The samplings were carried out by a fisherman provider (Prélèvements®) using a prawn net dredged with a mesh  
 131 size of 11 mm and dredging at a depth of 7 – 9 m. Water temperature, salinity and dissolved oxygen were  
 132 systematically recorded at each station during all samplings. Immediately after dredging, prawns were put in  
 133 30 L-plastic containers supplied with the natural brackish water of the sampling station, under oxygenation until  
 134 the return to the laboratory (i.e. approximatively 3 hours). The presence of the appendix *masculina* (i.e.  
 135 secondary sexual character) by an observation with binocular magnifier (8x) was used to select sexually mature  
 136 male prawns and to separate them from females (Fig 2). Males were kept in plastic containers under oxygenation  
 137 until analysis of sperm DNA damage, the next morning, as described in section 2.4.



138  
 139 **Figure 1.** Localization of the sampling stations along the Seine estuary between the Normandy bridge and the  
 140 Tancarville bridge (Normandie; France). NB1 = Normandie bridge; NB2 = Grestain abbey; NB3 = Grestain; Ri1  
 141 = Berville sur mer; RiR = Risle River; Ri2 = Blanc banc; TB1 = Pointe de la Roque and TB2 = Tancarville  
 142 bridge.





143

144 **Figure 2.** Binocular observation (8x) of the secondary sex characters, the appendix *masculina*, in  
 145 *Palaemon longirostris* at the second pleopode pair. The continuous arrow marks the appendix *interna* and the  
 146 hatched arrow marks the appendix *masculina*.

147

#### 148 2.2. Procedure of the sperm DNA damage analysis

149 Spermatozoa were extracted from the terminal ampullae before being transferred into 1.5 mL-microtube filled  
 150 with 300  $\mu$ L of artificial brackish water (*i.e.* salinity of 15) adjusted to the hemolymphatic osmolality of  
 151 *P. longirostris* (*i.e.* 610 – 650 mOsmol.kg<sup>-1</sup>). Spermatozoa were ripped by pipetting up and down until their  
 152 entire laceration. The mortality of spermatozoa suspension was assessed with the trypan blue test (*i.e.* 0.4 % w/v)  
 153 on KOVA® slides, using a photonic microscope (400x). Only the samples displaying a sperm viability  $\geq$  85 %  
 154 were used for the Comet assay. The procedure of the Comet assay was performed according to the methodology  
 155 developed by Singh et al. (1988) and previously adapted to Palaemonid prawns in Erraud et al. (2018a).

156

#### 157 2.3. Experiment of passive recovery in clean artificial sea water

158 Sixty adult male prawns from the sampling of January 2016 were used for this experiment. To respond to the  
 159 experimental design, the specimens from sampling stations Ri1 and Ri2 were used because high densities of  
 160 prawns, displaying similar level of spermatozoa DNA damage (see section 3.1), were found on these stations  
 161 during this period. At the beginning, 20 prawns were sacrificed to assess the initial sperm DNA integrity (T0).  
 162 The other forty prawns were distributed in groups of 5 prawns per 2 L-beaker containing 1 L of artificial  
 163 brackish-water (ABW) at salinity of 10, pH  $7.7 \pm 0.2$ , and  $12.0 \pm 0.3$  °C (*i.e.* approximating conditions found at  
 164 the sampling stations at this period) and kept in these conditions during 20 days. ABW was obtained by  
 165 dissolution of TETRA®Sea salt (*i.e.* salt used for marine aquarium maintenance) at a concentration of 12.3 g.L<sup>-1</sup>

166 (*i.e.* salinity of 10). Mortality was followed every day of this experiment. Every beaker was provided with  
167 additional aeration to maintain optimum conditions, and the water was monitored daily for nitrite ( $\text{NO}_2^-$ ) and  
168 nitrate ( $\text{NO}_3^-$ ) concentrations which were always included in low values (*i.e.* between 0.01 to 0.02  $\text{mg.L}^{-1}$  for  
169 nitrite and 5 to 9  $\text{mg.L}^{-1}$  of nitrate). The brackish water was renewed every 72 hours. Prawns were fed daily *ad*  
170 *libitum* with pellet B-Penaeus Grower RCE 1 (Le Gouessant®) according to manufacturer's recommendations  
171 during the experiment period. Batches of 4 beakers were stopped after 10 and 20 days of recovery (*i.e.* T10 and  
172 T20). Sperm samples from each surviving prawn were obtained and individually analysed as described in section  
173 2.4. The DNA integrity was measured only for the sperm samples displaying a cell viability  $\geq 85\%$  (*i.e.*  $n = 17$ ,  
174 12 and 14 for T0, T10 and T20, respectively).

175

#### 176 2.4. Experiment of active recovery by three successive forced spermatophore extraction / reformation

177 To ensure that the spermatophore turnover occurs with sperm showing a lower history of exposure, this  
178 experiment was conducted during the sexual latency. Seventy adult male prawns of the October 2016 sampling  
179 pooled from stations Ri1 and Ri2 were used for the same reasons than for the passive recovery (see section 2.2).  
180 Spermatophores for each male prawn were immediately extracted and the 20 sperm suspensions were randomly  
181 selected to obtain the T0. Just after the spermatophore extraction, prawns were distributed by 5 prawns per 2 L-  
182 beaker supplied with 1 L of ABW at salinity of 5, pH 7.9 and 13°C (*i.e.* approximating conditions found at the  
183 sampling stations at this period) and maintained for 30 days. ABW was obtained by dissolution of TETRA®Sea  
184 salt at a concentration of 6.1  $\text{g.L}^{-1}$  (*i.e.* salinity of 5). Fifteen days between each spermatophore extraction were  
185 necessary at this season to allow the formation of a new spermatophore in the terminal ampullae. The mortality  
186 of prawns was monitored every day. The maintenance of prawns was performed similar to that detailed in  
187 section 2.2. After 15 days and 30 days (*i.e.* T15 and T30), new extractions of spermatophores were performed on  
188 all prawns. Twenty sperm suspensions were randomly selected for DNA integrity analysis. The sperm samples  
189 of each prawn were obtained and individually analysed as described in section 2.4. The DNA integrity was  
190 measured only for the sperm samples displaying a cell survival rate  $\geq 85\%$  (*i.e.*  $n = 17$ , 17 and 12 for T0, T15  
191 and T30, respectively).

192

#### 193 2.5. Statistical analysis

194 Statistical analyses were performed with the R studio software v0.99.903 (RStudio Inc.). All results are

195 expressed as mean  $\pm$  standard deviation of all sperm suspensions for each condition. As normality and  
196 homoscedasticity was not respected for our comet assay data, only non-parametric tests were used. For DNA  
197 damage values of passive and active recovery, statistical comparisons of recovery times were assessed using the  
198 non-parametric Kruskal-Wallis rank ANOVA test, followed when significant by Wilcoxon rank sum test to  
199 identify groups that differed significantly from the control. The statistical significance level was set at 0.05. A  
200 maximum reference threshold (unilateral 95<sup>th</sup> percentile) of DNA damage was defined by the basis of data  
201 measured after active recovery (*i.e.* T15 and T30;  $n = 29$ ; see section 3.2).

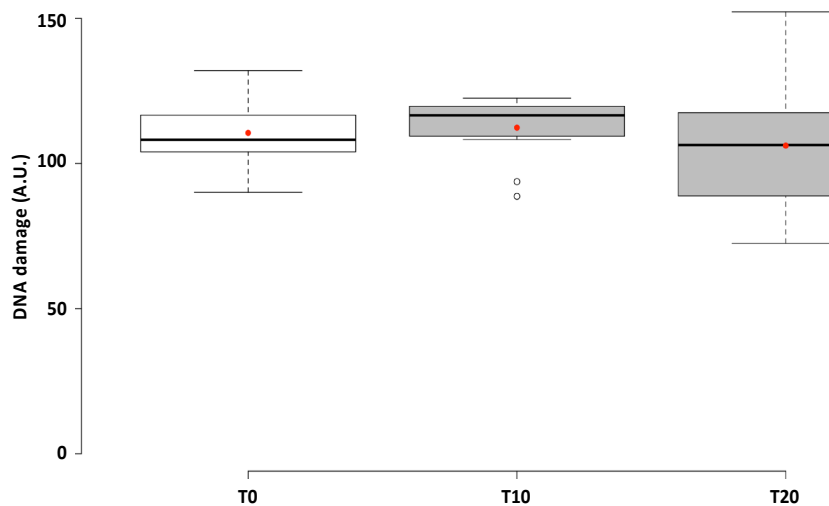
202 For each sampling campaign and each station, a pairwise comparison using a non-parametric Nemenyi rank sum  
203 test was used to assess the deviation of sperm DNA integrity from the defined baseline. A second non-parametric  
204 test, the Scheirer-Ray-Hare pairwise test, was used to assess the effects between stations and seasons.

205

### 206 3. Results

#### 207 3.1. Kinetics of DNA damage in spermatozoa of prawns from the Seine River during passive recovery

208 During this experiment, the prawn survival were 60 and 95 % in the batches of prawns stopped after 10 and 20  
209 days, respectively. The death was the result of cannibalism after moulting during the previous night. A slight but  
210 significant decrease of the sperm viability was observed after the first 10 days of this passive recovery  
211 (Wilcoxon rank sum test;  $p = 0.0004307$ ), but was not extended during the last 10 days (Wilcoxon rank sum  
212 test;  $p = 0.2605$ ) with a viability at T0, T10 and T20 of  $95.9 \pm 3.9$ ,  $90.3 \pm 3.8$  and  $93.3 \pm 6.4$  %, respectively.  
213 Fig. 3 shows the level of DNA damage measured in spermatozoa of the prawns sampled in the Seine estuary in  
214 January 2016 (*i.e.* T0;  $n = 17$  prawns) and after 10 and 20 days of passive recovery in clean ABW ( $n = 12$  and 14  
215 prawns, respectively). No significant decrease in the mean level of DNA damage was observed between T0, T10  
216 and T20 with levels reaching  $110.5 \pm 11.3$ ,  $112.3 \pm 10.7$  and  $106.1 \pm 22.7$  A.U. (*i.e.* Arbitrary Unit), respectively  
217 (Kruskal-Wallis rank test,  $p = 0.397$ ). However, it can be noted that an increase of the inter-individual variability  
218 at T20 days (*i.e.* Variation Coefficient VC = 21.3 %) was observed compared to the T0 and T10 days (*i.e.* 10.3  
219 and 9.6 %, respectively).



220

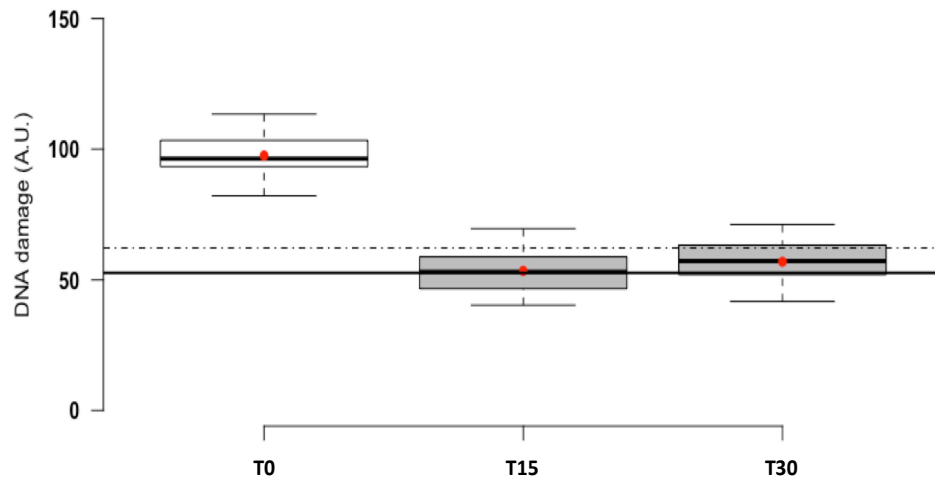
221 **Figure 3.** Sperm DNA damage measured using Comet assay in *Palaemon longirostris* specimens sampled in  
 222 Seine estuary, just before (*i.e.* T0;  $n = 17$ ) and after 10 and 20 days in laboratory recovery period in healthy  
 223 environment (T10 and T20;  $n = 12$  and  $14$ ). Results are shown in boxplot (*i.e.* the median, the first and the third  
 224 quartiles, the non-outliers range and the outliers), with the mean (red point).

225

### 226 3.2. Definition of reference values of DNA damage in spermatozoa of prawns in laboratory experiment

227 During this experiment, 21 prawns died which represent 30 % of the total number of prawns. Extraction of  
 228 spermatophores was the major source of mortality in this experiment with 7 and 8 dead prawns after the first and  
 229 second extractions, respectively. The 6 other dead prawns occurred after the moult as a result of cannibalism. A  
 230 slight but significant decrease of the cells viability was observed between T10 and T30 with viability at T0, T15  
 231 and T30 reaching  $97.0 \pm 2.0$ ,  $94.8 \pm 5.3$  and  $92.2 \pm 6.6$  %, respectively (Wilcoxon rank sum test;  $p = 0.002366$ ).  
 232 Fig. 4 presents the level of DNA damage measured in spermatozoa of prawns before (*i.e.* T0) and after the first  
 233 and second forced spermatophore extractions / reformations during a recovery phase in clean ABW (*i.e.* T15 and  
 234 T30). At T0, the mean level of DNA damage in spermatozoa of prawns has attained  $97.5 \pm 7.4$  A.U. After the  
 235 first reformation, at T15, a significant decrease of 54.8 % in the mean level of DNA damage was observed  
 236 reaching  $53.4 \pm 8.9$  A.U. (Wilcoxon rank sum test;  $p < 0.001$ ). In contrast, after the third extraction, a  
 237 stabilization of the mean level of DNA damage was observed between the first and the second reformation (*i.e.*  
 238  $57.0 \pm 9.1$  AU) (Wilcoxon rank sum test;  $p = 0.3033$ ). Hence, on the basis of the data T15 and T30 (*i.e.*  $n = 17$   
 239 and  $12$ , respectively), a baseline level was established, with a mean value of  $54.9 \pm 9.1$  A.U. and a more than  
 240 95 % unilateral confidence threshold of 69.7 A.U. This maximum damage threshold is based on the variation of

241 the 29 prawns' level of DNA damage at T15 and T30 days (*i.e.*  $n = 17$  and  $n = 12$ , respectively), which notably  
 242 presented a quite small inter-individual variability (Variation Coefficient - VC = 16.4 %).



243

244 **Figure 4.** Sperm DNA damage measured using Comet assay in *Palaemon longirostris* specimens sampled in  
 245 Seine estuary after 3 successive extractions of spermatophores performed just before (*i.e.* T0;  $n = 17$ ) and after  
 246 15 and 30 days of recovery under laboratory conditions in healthy medium (T15 and T30;  $n = 17$  and 12).  
 247 Results are shown in boxplot (*i.e.* the median, the first and the third quartiles, the non-outliers range and the  
 248 outliers), with the mean (red point). Continuous line represents the mean of DNA damage values recorded at T15  
 249 and T30 and the dashed line represents the unilateral 95 % confidence interval.

250

### 251 3.3. Sperm DNA damage within the prawn population of the Seine estuary

252 The seasonal variations of water temperature (*i.e.* from 5.7 to 14.8 °C), salinity (*i.e.* salinity from 3 to 16) and  
 253 dissolved oxygen (*i.e.* 5.1 – 8.6 ppm) observed at the sampling area during the different campaigns are  
 254 represented in Table 1. Fig. 5 presents the level of sperm DNA damage measured in native population of prawns  
 255 sampled at a maximum of seven stations located between the Normandy and the Tancarville bridges.

256

257

258

259

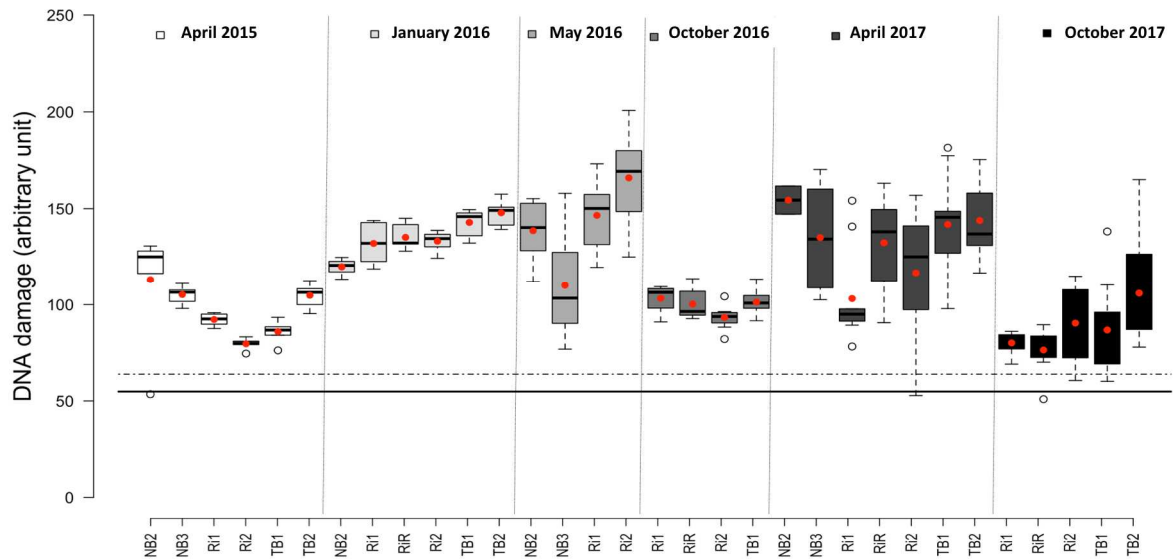
260

261 **Table 1.** Detailed informations concerning the different campaigns of sampling on the 8 stations from 2015 to  
 262 2017. ND = not determined.

| Sampling dates | Sites | T°C  | Salinity | Dissolved oxygen (ppm) | Dissolved oxygen (%) |
|----------------|-------|------|----------|------------------------|----------------------|
| 16/04/2015     | NB1   | 12.7 | 14       | ND                     | ND                   |
|                | NB2   | 12.5 | 16       | ND                     | ND                   |
|                | NB3   | 12.6 | 12       | ND                     | ND                   |
|                | Ri1   | 12.7 | 10       | ND                     | ND                   |
|                | RiR   | ND   | ND       | ND                     | ND                   |
|                | Ri2   | 12.9 | 8        | ND                     | ND                   |
|                | TB1   | 13.1 | 6        | ND                     | ND                   |
| TB2            | 13.3  | 3    | ND       | ND                     |                      |
| 21/01/2016     | NB1   | 5.7  | 17       | ND                     | ND                   |
|                | NB2   | 5.7  | 15       | ND                     | ND                   |
|                | NB3   | 5.7  | 12       | ND                     | ND                   |
|                | Ri1   | 5.8  | 9        | ND                     | ND                   |
|                | RiR   | 5.9  | 10       | ND                     | ND                   |
|                | Ri2   | 5.7  | 8        | ND                     | ND                   |
|                | TB1   | 5.8  | 8        | ND                     | ND                   |
| TB2            | 5.8   | 7    | ND       | ND                     |                      |
| 13/05/2016     | NB1   | 14.3 | 11       | 5.88                   | 82.3                 |
|                | NB2   | 14.8 | 9        | 6.46                   | 80.4                 |
|                | NB3   | 14.7 | 7        | 5.27                   | 79.6                 |
|                | Ri1   | 14.1 | 6        | 5.17                   | 76.5                 |
|                | RiR   | 14.5 | 5        | 5.93                   | 81.4                 |
|                | Ri2   | 14.3 | 5        | 5.15                   | 78.3                 |
|                | TB1   | 14.1 | 4        | 6.2                    | 79.0                 |
| TB2            | 14.7  | 3    | 5.61     | 80.0                   |                      |
| 25/10/2016     | NB1   | 13.7 | 25       | 7.21                   | 81.4                 |
|                | NB2   | 13.1 | 19       | 7.52                   | 83.6                 |
|                | NB3   | 12.8 | 16       | 7.34                   | 82.5                 |
|                | Ri1   | 12.9 | 14       | 7.63                   | 86.8                 |
|                | RiR   | 12.7 | 5        | 8.02                   | 90.2                 |
|                | Ri2   | 12.9 | 6        | 7.83                   | 87.4                 |
|                | TB1   | 13.0 | 5        | 7.65                   | 85.9                 |
| TB2            | 12.9  | 4    | 7.47     | 81.7                   |                      |
| 17/04/2017     | NB1   | 12.5 | 14       | 8.60                   | 92.0                 |
|                | NB2   | 12.5 | 13       | 8.70                   | 95.8                 |
|                | NB3   | 12.4 | 12       | 8.20                   | 90.6                 |
|                | Ri1   | 12.5 | 10       | 8.17                   | 90.1                 |
|                | RiR   | 13.2 | 4        | 8.58                   | 92.0                 |
|                | Ri2   | 12.9 | 6        | 7.83                   | 88.0                 |
|                | TB1   | 13.1 | 3        | 8.02                   | 90.0                 |
| TB2            | 13.1  | 3    | 7.99     | 88.0                   |                      |
| 25/10/2017     | NB1   | 14   | 19       | 6.95                   | 85.3                 |
|                | NB2   | 13.9 | 17       | 7.58                   | 83.1                 |
|                | NB3   | 13.7 | 16       | 7.21                   | 83.2                 |
|                | Ri1   | 13.6 | 15       | 7.64                   | 84.4                 |
|                | RiR   | 14.3 | 6        | 8.01                   | 89.5                 |
|                | Ri2   | 14   | 7        | 7.73                   | 87.2                 |
|                | TB1   | 14.2 | 10       | 7.54                   | 82.2                 |
| TB2            | 14.3  | 6    | 7.61     | 83.9                   |                      |

263

264



265

266 **Figure 5.** Sperm DNA damage measured using Comet assay in *Palaemon longirostris* specimens sampled in a  
 267 maximum of 7 stations along the mesohaline part of the Seine estuary during 6 campaigns in 2015, 2016 and  
 268 2017. Results are shown in boxplot (*i.e.* the median, the first and the third quartiles, the non-outliers range and  
 269 the outliers), with the mean (red point).  $n = 10$  specimens per station.

270

271 Prawns sampled in all stations and campaigns presented mean DNA damage levels above the maximum damage  
 272 threshold of 69.7 A.U. defined in the previous laboratory experiment (see section 3.2). Considering the mean  
 273 level of all stations for each sampling campaign, the lowest levels of sperm DNA damage were observed in April  
 274 2015, October 2016 and October 2017 with values reaching  $96.8 \pm 16.8$ ,  $99.0 \pm 7.7$  and  $88.7 \pm 21.7$  A.U. (*i.e.*  
 275 38.9, 42.1 and 27.3 % above the maximum threshold), respectively. Contrarily, the highest levels of sperm DNA  
 276 damage were observed in January 2016, May 2016 and April 2017 displaying levels of  $135.2 \pm 11.1$ ,  $137.4 \pm$   
 277  $28.2$  and  $129.3 \pm 21.7$  A.U. (*i.e.* 94.0, 97.1 and 85.5 % above the threshold value), respectively. Considering now  
 278 the inter-site variability of each sampling campaign, during the spring (*i.e.* April 2015, May 2016 and April  
 279 2017), it can be underlined that the prawns showed an inter-site variability with a higher mean level of DNA  
 280 damage at each sampling station extremity considering the location of prawns (Nemenyi rank sum test,  $p <$   
 281 0.05). Indeed, the lowest levels of DNA damage were observed in the middle of the studied area, Stations Ri2,  
 282 NB3 and Ri1 displaying a minimum of  $79.6 \pm 3.2$ ,  $110.1 \pm 27.2$  and  $103.2 \pm 24.2$  A.U. in April 2015, May 2016,  
 283 April 2017, respectively. Conversely, during the sampling of January 2016 and October 2016 no significant  
 284 difference was observed between stations (Nemenyi rank sum test,  $p > 0.05$ ). Moreover, during May 2016, April  
 285 2017 and October 2017, a significant inter-individual variability at all stations has been observed (*i.e.* Variation



286 Coefficient % from 10.9 to 24.7 %, from 6.7 to 29.8 % and from 7.6 to 27.2 %, respectively) which is not the  
287 case in April 2015, January 2016 and October 2016 (*i.e.* VC % from 3.2 to 7.5 %, from 3.5 to 8.4 % and from  
288 6.4 to 8.1 %, respectively). *In fine*, a significant variability on the level of sperm DNA damage between-month  
289 was observed (Scheirer-Ray-Hare pairwise test;  $p = 2.220446e-16$ ).

290

#### 291 4. Discussion

##### 292 4.1. Relevance of the different recovery procedures as an alternative to the availability of a reference population

293 In this present study, the ability of *Palaemon longirostris* spermatozoa to recover sperm DNA damage was  
294 assessed from indigenous specimens of the Seine estuary (*i.e.* naturally exposed to environmental contamination)  
295 after transfer in the laboratory conditions, during a 20 days-period. In the case of the majority of spermatozoa,  
296 they are known to be devoid of DNA repair mechanisms (Aitken et al., 2004). However, crustaceans  
297 spermatozoa, and more precisely Palaemonidae prawns, present a number of morphological and structural  
298 particularities (*e.g.* afflagellate, no systematic presence of an acrosome, and mainly a totally decondensed  
299 chromatin; Braga et al., 2013), compared with the other ent-aqua sperm, which seems closer of somatic cells.  
300 Prawns sampled in the Seine estuary (*i.e.* T0) displayed high sperm DNA damage levels compared to the  
301 baseline recently described in the coastal species *Palaemon serratus* from a reference station of the Seine Bay  
302 (*i.e.* Yport;  $52.6 \pm 5.6$  A.U. with the upper 95% unilateral confidence threshold at 61.7 A.U; Erraud et al., 2018).  
303 After 10 and 20 days of passive recovery in *P. longirostris*, the level of DNA damage was persistent over time  
304 (*i.e.*  $112.3 \pm 10.7$  and  $106.1 \pm 22.7$  A.U, respectively). This strategy to reach the baseline level of DNA damage  
305 for its use in biomonitoring program seems not to be operational. This result suggested a lack of DNA repair  
306 mechanisms in *Palaemon* sp spermatozoa and is coherent with previous reports in another crustacean group.  
307 Indeed, Lacaze et al. (2011b) observed no DNA repair in the sperm of the freshwater *Gammarus fossarum* after  
308 an initial exposure to the model genotoxicant methyl methane sulfonate for 5 days, and 4 days of recovery in  
309 clean water. The lack of recovery of the sperm DNA damage in *P. longirostris* reinforces its relevance in the  
310 environmental survey. Indeed, to be useful in this field, a biomarker has to be representative from stress over  
311 time (Wu et al. 2005). In this way, the capability of a fast recovering biomarker represents a real disadvantage  
312 for monitoring applications by reflecting only a recent pollution episode and not providing the time integration  
313 of contamination effects (Sanchez et al., 2008; Webb and Gagnon, 2013). Without repair mechanisms, the DNA  
314 integrity will depend on the cells sensitivity, genotoxic pressure and time of exposure. Although spermatogenesis



315 in the majority of crustaceans is performed within the testis, many later stages of this process are completed upon  
316 entering the testicular lumen and the proximal part of the vas deferens to assume complete morphological sperm  
317 characteristics (Subramoniam, 2016). Starting from this statement, an exposure of the spermatogenic line cells within  
318 the testicular lumen or the vas deferens could still display DNA repair mechanisms and consequently show less  
319 significant DNA damage than spermatozoa exposed in the spermatophore. Lacaze *et al.* (2011a) demonstrated  
320 that the sensitivity of Gammarid sperm DNA integrity could depend on the spermatogenesis stage. The authors  
321 observed the highest level of DNA damage in spermatozoa collected in sexual mature males in comparison to  
322 spermatozoa from males in the early stage of spermatogenesis. To ensure the renewal of the mature spermatozoa  
323 stock which would be unexposed, we forced the formation of new spermatophores by a manual extraction during  
324 the sexual rest period (*i.e.* with the vas deferens empty). In the present work, we defined that 15 days was  
325 necessary for *P. longirostris* to renew a spermatophore after a forced extraction. This methodology was already  
326 used in three Peneidae species in aquaculture to assess the time necessary for the renewal of spermatophores  
327 after a manual extraction and after a natural mating session (Leung-trujillo and Lawrence, 1991). In these  
328 tropical species, a range from two days to seven days was necessary depending on the species. The time of  
329 renewal in *P. longirostris* was longer than for Peneidae prawns; this does not seem surprising since the  
330 metabolism is slowed down in temperate species. After the first renewal of spermatophores (*i.e.* T15), the level  
331 of DNA damage decreases sharply to attain a relatively low level. Ultimately, a settling down of the sperm level  
332 of DNA damage was observed after the second reformation (*i.e.* T30). A mean value of  $54.9 \pm 9.1$  A.U. and a  
333 maximum threshold corresponding to the unilateral 95 % confidence intervals of 69.7 A.U. was generated on the  
334 basis of the compilation of the levels of DNA damage recorded at T15 and T30 days ( $n = 17$  and 12 prawns,  
335 respectively). This level of DNA damage seems coherent with the reference distribution defined in a previous  
336 study (based on a monthly chronological data set of two years at a reference station) in the coastal species  
337 *Palaemon serratus* (*i.e.* a mean  $\pm$  SD of  $52.6 \pm 5.6$  A.U. and a IC 95% of 61.7 A.U.; Erraud *et al.* 2018). Despite  
338 its apparent limitation in terms of replication of conditions between the laboratory and field conditions (*i.e.* water  
339 chemistry, circadian cycles and food), the results of this experiment seem to be coherent with those obtained in  
340 realistic conditions in the coastal species for which no effect of confounding factors (*i.e.* season, temperature,  
341 age and moult-stage) was observed. This observation improves the relevance of the procedure for an active  
342 recovery to reach a baseline level of sperm DNA integrity in Palaemonidae prawns. Consequently, a DNA  
343 damage level above the threshold could be interpreted as a modulation, resulting from an exposure to  
344 contaminants.

345

346 4.2. Focus on sperm DNA integrity of the *Palaemon longirostris* population within the Seine estuary

347 The levels of sperm DNA damage were measured in prawns coming from a maximum of 7 different stations  
348 along the salinity gradient of the Seine estuary, during April 2015, January 2016, May 2016, October 2016, April  
349 2017 and October 2017. All investigated stations during the 6 sampling campaigns exhibited high levels of  
350 sperm DNA damage with percentages of induction above the maximum threshold (previously described in  
351 section 3.2 & 4.1) ranging from 27.3 % in autumn 2017 (*i.e.* October) to 97.1 % in spring 2017 (*i.e.* April),  
352 highlighting the abnormally elevated response of this biomarker in this part of the Seine estuary. If we compare  
353 these results with the ones obtained on the coastal species *P. serratus* in different stations of the Seine Bay in  
354 autumn 2015 and 2016 (*i.e.* October), in most cases the percentage of induction is higher in the Seine estuary  
355 than in the three most impacted stations of the Seine Bay located at the north of the mouth of the river (*i.e.* 16.3  
356 to 29.5 %) (Erraud et al. 2018). This observation is in accordance with the geographical gradient of  
357 contamination which is diluted from the estuary to the coastline water bodies.

358 It may be expected in the estuary biotope, which displays considerable physico-chemical fluctuations, that  
359 environmental conditions could impact the basal level of a biological response, confusing biomarker  
360 interpretation. The influence of environmental factors is generally studied in reference station (*i.e.*  
361 uncontaminated) or in controlled laboratory condition (*i.e.* in a lesser environmental realism) to avoid all  
362 agonistic or antagonistic effects of pollution during the interpretation (*e.g.* Xuereb et al., 2009; Lacaze et al.,  
363 2011a; Coulaud 2011). In this work, the correlations between temperature, dissolved oxygen or salinity, and  
364 DNA damage were tested, and no significant effect was observed (data not shown). However, regarding the high  
365 level of DNA damage observed during this study and the degraded chemical quality of the Seine estuary, it  
366 seemed no relevant to use this data to validate the absence of physical factor influence. Nevertheless, the return  
367 on previous experiment in the coastal species *Palaemon serratus* added to the observations reported in the  
368 literature lead us to exclude incidence of these confounding factors in the range of variations observed in the  
369 present study. Indeed, precedent works have shown that the range of temperature measured in the living  
370 environment of prawns seems to have no influence on the baseline of sperm DNA damage for crustaceans, as in  
371 the common prawn *P. serratus* (Erraud et al., 2018b) or the freshwater gammarid *G. fossarum* (Lacaze et al.,  
372 2011b). In regard to the salinity or dissolved oxygen level, to our knowledge, no information exists concerning  
373 the influence of these two abiotic factors on the baseline of sperm DNA damage. However, Sing and Harlt

374 (2012), facilitated by assessing the impact of confounding factors in estuarine ecosystems, demonstrated in  
375 laboratory experiments that salinity from 8 to 32 was not a confounding factor for DNA integrity in gill cells and  
376 haemocytes in *M. edulis*. Moreover, *P. longirostris* is considered as an hyper euryhaline species which allow to  
377 conserve a great homeostasis condition facing salinity's variations (Campbell & Jones, 1989). So, it is difficult to  
378 believe that salinity can have an effect on sperm DNA damage levels in the range of salinity observed during the  
379 present study. Concerning the level of dissolved oxygen, an example of induced DNA strand breaks has already  
380 been shown in gill cells of the rainbow trout *Oncorhynchus mykiss* (Liepelt et al. 1995) and the common carp  
381 *Cyprinus carpio* (Mustafa et al. 2011), but only at the hypoxia stage (*i.e.*  $< 3 \text{ mg.L}^{-1}$ ). During our different  
382 sampling campaigns, the lowest concentration of dissolved oxygen was observed during the spring 2016 (*i.e.*  
383 May 2016) with values ranging from 5.2 to 6.5  $\text{mg.L}^{-1}$  which are not considered to be low values. These punctual  
384 measurements are corroborated by dissolved oxygen data collected by the SYNAPSE network (*i.e.* continuous  
385 monitoring of physiochemical parameters in the Seine River) which underlined the lack of anoxic episodes  
386 throughout the years of study (*i.e.* values upper than 5  $\text{mg.L}^{-1}$ ) (Romero et al., 2016).

387 Different patterns of sperm DNA damage were observed according to the season. In the current state of  
388 knowledge relative to the ecophysiology of *P. longirostris* and to the hydro-sedimentology and contamination of  
389 the Seine estuary (*i.e.* data generated at larger spatial and temporal frequencies), the fine interpretation of these  
390 patterns remains somewhat speculative. For example, the highest levels of DNA integrity of *P. longirostris*  
391 spermatozoa were observed during January 2016, May 2016 and April 2017. These observations could be  
392 explained by the high-water level period (*i.e.* from December to April) and consequently by the contamination  
393 loading from the river effluents and leaching of flood soils. In contrast, in October 2016 and October 2017, the  
394 level of the DNA integrity was lower, which could be in relation with the low-water level in accordance with the  
395 upwelling of coastal water in the investigated area. In other way, in January and October 2016, the levels of  
396 damage recorded in the different stations were close, displaying a low inter-individual variation coefficient (*i.e.*  
397 from 7 to 8 %). Conversely, notable inter-station variabilities were observed during the spring campaigns (*i.e.*  
398 April 2015, May 2016 and April 2017); the highest levels of DNA damage were measured at both extremities of  
399 the sampling area. It can be hypothesized that these fluctuations could be the result of local chemical pressure or  
400 an effect of the breeding period of the white prawn. It was shown in Palaemonidae species (*Macrobrachium*  
401 *nobili* and *Macrobrachium rosenbergi*), that during this period a single male could ensure the fertilization of  
402 several females in a row (Balasundaram & Pandian, 1982; Ling, 1967). So, it could be hypothesized that the  
403 sexual activity of male specimens accentuates the turnover of sperm stock, acting as an active recovery. The

404 lowest level of DNA observed in the middle part of the area could be explained by a more intense sexual activity  
405 (Aurousseau, 1984). In the same way, the more pronounced inter-individual variability could be the result of the  
406 temporality of reproduction events that could differ between specimens.

407 Further research must be performed to gain a better understanding of these spatial and temporal variations on the  
408 toxicological response and lead to a survey protocol which is as relevant as possible. Anyway, in the current  
409 situation, the global work conducted on the genus *Palaemon* sp., allowed to quantify a genotoxic pressure within  
410 a transition waterbody regarding a robust reference value, which constitute an important challenge for the  
411 application and the interpretation of biomarkers in fluctuating systems as estuaries.

412

### 413 **Conclusion**

414 The use of passive recovery in controlled laboratory conditions showed the lack of DNA repair mechanisms in  
415 *Palaemon* sperm, underlining the ability of the sperm DNA damage measurement for integrating the history of  
416 exposure to toxicological stress. Conversely, the active recovery (in the same conditions) appeared to be a  
417 relevant alternative strategy to define a baseline for this biomarker in an estuarine palaemonid prawn. This  
418 baseline of *Palaemon longirostris* was perfectly in accordance with the reference distribution defined in the  
419 coastal species *Palaemon serratus* suggesting the possibility of using an inter-species reference distribution to  
420 invest the continuum estuary/littoral of the Seine Bay. Although this study was a preliminary step, the use of this  
421 baseline demonstrated the abnormal level of sperm DNA damage within the autochthonous *P. longirostris*  
422 population of the Seine estuary during six campaigns from 2015 to 2017. However, further studies could be done  
423 to give more precision to the environmental diagnosis. For example, deployment of an active biomonitoring  
424 approach based on the transplantation of prawns into caging systems coupled to physico-chemical data loggers  
425 and chemical integrative samplers could lead to a better control of the *in situ* exposure conditions and  
426 consequently to a more accurate understanding of the biomarker fluctuations.

427

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432

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| SAMPLING DATES | SITES | T°C  | SALINITY | DISSOLVED OXYGEN (PPM) | DISSOLVED OXYGEN (%) |
|----------------|-------|------|----------|------------------------|----------------------|
| 16/04/2015     | NB1   | 12.7 | 14       | ND                     | ND                   |
|                | NB2   | 12.5 | 16       | ND                     | ND                   |
|                | NB3   | 12.6 | 12       | ND                     | ND                   |
|                | Ri1   | 12.7 | 10       | ND                     | ND                   |
|                | RiR   | ND   | ND       | ND                     | ND                   |
|                | Ri2   | 12.9 | 8        | ND                     | ND                   |
|                | TB1   | 13.1 | 6        | ND                     | ND                   |
|                | TB2   | 13.3 | 3        | ND                     | ND                   |
| 21/01/2016     | NB1   | 5.7  | 17       | ND                     | ND                   |
|                | NB2   | 5.7  | 15       | ND                     | ND                   |
|                | NB3   | 5.7  | 12       | ND                     | ND                   |
|                | Ri1   | 5.8  | 9        | ND                     | ND                   |
|                | RiR   | 5.9  | 10       | ND                     | ND                   |
|                | Ri2   | 5.7  | 8        | ND                     | ND                   |
|                | TB1   | 5.8  | 8        | ND                     | ND                   |
|                | TB2   | 5.8  | 7        | ND                     | ND                   |
| 13/05/2016     | NB1   | 14.3 | 11       | 5.88                   | 82.3                 |
|                | NB2   | 14.8 | 9        | 6.46                   | 80.4                 |
|                | NB3   | 14.7 | 7        | 5.27                   | 79.6                 |
|                | Ri1   | 14.1 | 6        | 5.17                   | 76.5                 |
|                | RiR   | 14.5 | 5        | 5.93                   | 81.4                 |
|                | Ri2   | 14.3 | 5        | 5.15                   | 78.3                 |
|                | TB1   | 14.1 | 4        | 6.2                    | 79.0                 |
|                | TB2   | 14.7 | 3        | 5.61                   | 80.0                 |
| 25/10/2016     | NB1   | 13.7 | 25       | 7.21                   | 81.4                 |
|                | NB2   | 13.1 | 19       | 7.52                   | 83.6                 |
|                | NB3   | 12.8 | 16       | 7.34                   | 82.5                 |
|                | Ri1   | 12.9 | 14       | 7.63                   | 86.8                 |
|                | RiR   | 12.7 | 5        | 8.02                   | 90.2                 |
|                | Ri2   | 12.9 | 6        | 7.83                   | 87.4                 |
|                | TB1   | 13.0 | 5        | 7.65                   | 85.9                 |
|                | TB2   | 12.9 | 4        | 7.47                   | 81.7                 |
| 17/04/2017     | NB1   | 12.5 | 14       | 8.60                   | 92.0                 |
|                | NB2   | 12.5 | 13       | 8.70                   | 95.8                 |
|                | NB3   | 12.4 | 12       | 8.20                   | 90.6                 |
|                | Ri1   | 12.5 | 10       | 8.17                   | 90.1                 |
|                | RiR   | 13.2 | 4        | 8.58                   | 92.0                 |
|                | Ri2   | 12.9 | 6        | 7.83                   | 88.0                 |
|                | TB1   | 13.1 | 3        | 8.02                   | 90.0                 |
|                | TB2   | 13.1 | 3        | 7.99                   | 88.0                 |
| 25/10/2017     | NB1   | 14   | 19       | 6.95                   | 85.3                 |
|                | NB2   | 13.9 | 17       | 7.58                   | 83.1                 |
|                | NB3   | 13.7 | 16       | 7.21                   | 83.2                 |
|                | Ri1   | 13.6 | 15       | 7.64                   | 84.4                 |
|                | RiR   | 14.3 | 6        | 8.01                   | 89.5                 |
|                | Ri2   | 14   | 7        | 7.73                   | 87.2                 |
|                | TB1   | 14.2 | 10       | 7.54                   | 82.2                 |
|                | TB2   | 14.3 | 6        | 7.61                   | 83.9                 |

**Highlights**

- Alternative strategy to propose a baseline of sperm DNA integrity in *P. longirostris*
- Persistence of sperm DNA damages during 20 days after the toxic stress cancelation
- Relevance of a significant threshold to discriminate abnormal genotoxic pressure

ACCEPTED MANUSCRIPT