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## Use of sperm DNA integrity as a marker for exposure to contamination in *Palaemon serratus* (Pennant 1777): Intrinsic variability, baseline level and in situ deployment

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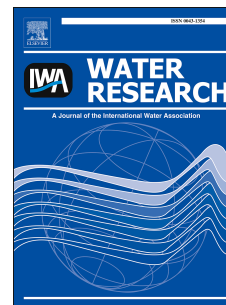
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# Accepted Manuscript

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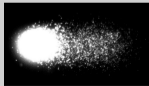
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# Sperm DNA integrity in Palaemonid prawns



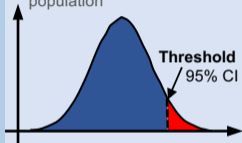
Unistellate sperm cell



Comet assay

## 1. Characterization of the natural variability ☆

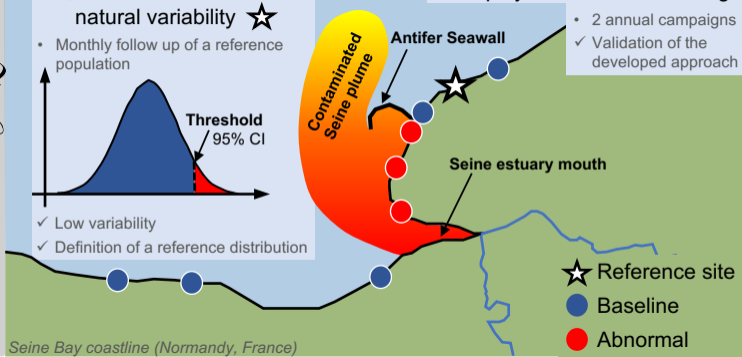
- Monthly follow up of a reference population



- ✓ Low variability
- ✓ Definition of a reference distribution

## 2. Deployment in biomonitoring

- 2 annual campaigns
- ✓ Validation of the developed approach



Seine Bay coastline (Normandy, France)

- ☆ Reference site
- Baseline
- Abnormal

1 **Use of sperm DNA integrity as a marker for exposure to**  
2 **contamination in *Palaemon serratus* (Pennant 1777):**  
3 **intrinsic variability, baseline level and *in situ* deployment.**

4  
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17 **Abstract**

18 In a previous study, the Comet assay was optimized for *Palaemon serratus* prawns in order to propose a biomarker for sperm quality in this  
19 species. However, better knowledge of its basal level and its natural variability, related to intrinsic biotic and environmental abiotic factors, is  
20 required before any relevant use of this biomarker in the field. To fulfill this goal, the present study proceeded in three steps: (i) the temporal  
21 variability of DNA integrity was followed monthly in a reference population over a 2-year period, (ii) the correlation between the main  
22 intrinsic biotic (i.e. size, weight and molting stage) and abiotic factors (i.e. water temperature) were recorded in the field, and the basal DNA  
23 integrity was assessed in order to scrutinize any confounding influence of factors unrelated to toxic response, (iii) the baseline level was used  
24 to discriminate biomarker response among different stations displaying contrasting contamination levels. The results of the two-year  
25 monitoring in the reference population revealed no correlation between the levels of spermatozoa DNA damage and temperature, body size,  
26 weight or molting stage. Only a slight variability between monthly samplings was detected. On the basis of these field-collected data, we  
27 defined a reference distribution (i.e.  $52.6 \pm 5.6$  A.U) with a threshold value (i.e. 61.7 A.U). Finally, this threshold value proved its relevance  
28 to discriminate among stations with contrasting pollution levels around the Seine Bay. Indeed, the results suggest significant DNA damage in  
29 populations nearest the Seine estuary, a major source of contaminants in the Bay, and a lower effect in populations further away from the  
30 estuary. The overall conclusion was that the Comet assay on *P. serratus* spermatozoa could be a useful tool for the monitoring of the  
31 toxicological print within sperm and main globally the contamination exposure of crustaceans in marine waters.

32  
33 **Key words: Comet assay, Environmental genotoxicity, Biomonitoring, Reference, Threshold value,**  
34 **Crustacean.**

## 35 1. Introduction

36 Pollution released from industrial wastewater, farming, and urban sources is a major threat to organisms living in  
37 the aquatic environment. Nowadays, estuarine and marine areas are one of the main preoccupations of aquatic  
38 ecotoxicologists; due to their ultimate destination position for most anthropogenic compounds, they are  
39 subjected to contamination from point and diffuse sources (Tappin and Millward, 2015). From a toxicological  
40 point of view, biocenotic approaches and chemical analyses (e.g. sediment and biota), which are recommended  
41 by the Water Framework Directive 2000/60/EC, appear to be limited in their capacity to identify contamination  
42 effects on aquatic populations. To address this constraint, the UE Marine Strategy Framework Directive  
43 2008/56/EC introduced the development of biomarkers with the view to their deployment in transitional and  
44 coastal waters. Indeed, biomarkers are early-warning tools assessing a causal relationship between the exposure  
45 to chemicals and the impacts on organisms, including factors such as bioavailability and mixture effects of  
46 known and unknown chemicals (Hanson et al., 2010). Biomarkers should be reliable, robust, easily applicable,  
47 only modulated by contaminants, and predictors of adverse impacts on populations and communities. In reality,  
48 almost all biomarkers are influenced by abiotic factors such as water temperature, salinity, pH and dissolved  
49 oxygen ( Buschini et al., 2003; Bolognesi et al., 2004; Leinio and Lehtonen, 2005; Pfeifer et al., 2005) or  
50 intrinsic variables linked to age, sex or reproductive stage (Jha, 2008; Lacaze et al., 2011a; Sanchez et al., 2008;  
51 Sheehan and Power, 1999; Wiklund and Sundelin, 2004; Xuereb et al., 2009). Because of these confounding  
52 factors, biomarkers are still not often routinely employed in biomonitoring surveys, particularly in studies on a  
53 spatial or temporal large scale. The major part of *in situ* application with biomarkers is generally based on the  
54 comparison between a reference site and an impacted site (e.g. upstream and downstream comparisons)  
55 (Flammarion et al., 2002; Flammarion and Garric, 1997). This (i) imposes similar physicochemical conditions  
56 between stations in absence of contamination (Underwood, 2000) and (ii) consequently limits the assessment in  
57 punctual studies and local areas. Alternatively, some solutions have been proposed to control the influence of  
58 both biotic and environmental factors on the response of biomarkers, and make possible their deployment within  
59 field surveys on a larger scale. Among them, the assessment of natural variability to propose a basal reference  
60 while taking into account a spatiotemporal change has proved its effectiveness (Barrick et al., 2016; Hagger et  
61 al., 2008; Hanson et al., 2011; Jubeaux et al., 2012; Lacaze et al., 2011a; Xuereb et al., 2009). This methodology  
62 makes it possible to (i) compare sites without a reference site, (ii) reveal the exposure of organisms to one or  
63 several contaminants in low contrasted or contaminated situations and (iii) prevent false negatives. For the  
64 estimation of such ranges, a monitoring of several reference sites in different seasons is recommended. The

65 OSPAR convention in 2013 has recommended that organisms should be collected from reference sites for at  
66 least two seasonal cycles to assess the influence of confounding factors (Amiard-Triquet et al. 2015).

67 Among biomarkers for damage, genotoxicity biomarkers are considered as integrated tools, able to provide  
68 complementary information to chemical and ecological analyses for field monitoring (Lacaze et al., 2011a).  
69 Indeed, contaminants described as potentially genotoxic would represent one-third of the anthropogenic  
70 compounds released into the marine environment (Claxton et al., 1998). Interaction of these compounds on the  
71 reproduction process may have an even greater impact at the population level than carcinogenic effects for  
72 organisms (Aitken and De Iuliis, 2007). In germ line cells, spermatozoa proved to be sensitive to water  
73 contamination because of their inability to prevent oxidative stress and to repair DNA damage. These  
74 particularities make them a more integrated cellular model of chemical exposure than oocytes (Aitken et al.,  
75 2004; Aitken and Baker, 2006; Lacaze et al., 2010; Lee and Steinert, 2003; Lewis and Galloway, 2009; Santos et  
76 al., 2014). Moreover, several authors have already demonstrated the relationship between genotoxicity in sperm  
77 and reproductive impairment, in invertebrates (Lacaze et al., 2011a; Lewis & Galloway., 2009) as well as in fish  
78 species (Devaux et al., 2011; Santos et al., 2013ab). These studies highlighted the main interest of including a  
79 biomarker of genotoxicity measured in sperm for environmental monitoring and ecological risk assessment. The  
80 Comet assay or Single-cell gel electrophoresis assay (SCGE) has become one of the most widely used for  
81 detecting DNA strand breaks in aquatic animals (Cotelle and Fe, 1999; Frenzilli et al., 2009; Jha, 2008; Lacaze  
82 et al, 2011; Lee and Steinert, 2003; Martins and Costa, 2015; Mitchelmore and Chipman, 1998). Furthermore,  
83 Comet assay on spermatozoa for environmental monitoring has been recommended in many reviews (Jha, 2008;  
84 Speit et al., 2009; Villani et al., 2010).

85 As a member of the crustacean family, Palaemonid prawns have been commonly used as a relevant sentinel  
86 species for assessing the health conditions of estuaries and coastal systems (Bocquene et al., 1995; Frasco et al.,  
87 2008; Key et al., 2006). These species are relatively easy to identify, manipulate and maintain in the laboratory  
88 or to use for *in situ* biosurveys. *Palaemon* genus is widespread and common in the coastal and estuarine waters  
89 of Western Europe, where they are often found in high density (Campillo, 1975). Moreover, these prawns are an  
90 important trophic component for many fish and other crustacean species, including commercially valuable ones,  
91 and play a major part in the detritus breakdown process, being primary and secondary consumers (Anderson,  
92 1985). A particular interest has recently been attributed to the *Palaemon serratus* species, which is a coastal  
93 species with a large distribution around the European coast (North Sea, English Channel, Atlantic and  
94 Mediterranean coast; Campillo, 1975).

95 Our work aims to develop the Comet assay on Palaemonid spermatozoa and propose it as a reliable and robust  
96 biomarker for the surveys of the European coastal and estuarine water bodies. As recommended by Azqueta and  
97 Collins (2013), a preliminary study related to the methodological optimization of the Comet assay protocol was  
98 performed in *P. serratus* (Erraud et al., 2017). This step, which is unavoidable before any deployment in field  
99 studies, led us to propose a procedure for collecting a homogeneous population of mature spermatozoa and  
100 measuring their DNA integrity. Palaemonid sperm turned out to be sensitive to different genotoxic pathways.  
101 Henceforth, the aim of the present study was (i) to develop a procedure to obtain the lowest variability of  
102 biomarker response related to intrinsic biotic factors, and (ii) to define reference values of our genotoxicity  
103 biomarker taking into account the effect of environmental confounding factors. For that, the inter-individual and  
104 seasonal variability of the basal level of sperm DNA damage was investigated during a two-year period within a  
105 wild population of prawns in a reference site (i.e. Yport), in order to propose a reference distribution and a  
106 threshold value for this biological measurement. In the second step, sperm DNA damage was measured during  
107 two campaigns of biomonitoring within populations in different stations of the Seine bay displaying contrasting  
108 degrees of contamination, in order to assess the relevance of this reference distribution to discriminate toxic  
109 effect.

110

## 111 2. Materials and methods

### 112 2.1. Localisation of studied area

113 The mouth of the Seine (Normandy, France) is marked by the discharge of a wide diversity of chemical  
114 contaminants (i.e. drained by the Seine River), most of which are associated to the particles of the sedimentary  
115 plume. The contamination is transported by the longshore drift along the coastline mainly in a northerly direction  
116 (Augris et al., 2004), up to the seawall of Antifer Harbor. This 3-kilometer long transverse structure deflects the  
117 contaminated plume offshore, creating a sedimentation area upstream of the seawall (i.e. south of Antifer  
118 Harbor) (Brivois et al., 2015).

119 The contamination gradients from the Seine estuary toward both the north and the south of the bay are well  
120 illustrated by the ROCCH data records (Chemical Contamination Observation Network;  
121 <http://www.ifremer.fr/envlit/>) synthetized in Table 1. This table indicates the annual mean (i.e. 2015 and 2016)  
122 of cumulated concentrations of polychlorobiphenyl congeners (i.e. CB138; CB180; CB156; CB28; CB52;  
123 CB105; CB153; et le CB101), polycyclic aromatic hydrocarbons (i.e. Benzo(b)fluoranthene; Chrysene;  
124 Dibenzo(a,h)anthracene; Fluoranthene; Indeno(1,2,3-cd)pyrene; Anthracene; Phenanthrene; Pyrene;

125 Benzo(g,h,i)perylene; Benzo(k)fluoranthene; Benzo(a)pyrene; Benzo(a)anthracene) and seven metals (namely.  
126 Zinc, Cadmium, Copper, Mercury, Silver, Nickel and Lead) measured in soft tissues of mussels sampled from  
127 both sides of the mouth of the Seine. A contamination index was calculated to facilitate the appreciation of the  
128 contamination level of the different studied ROCCH stations. On the basis of rank transformation according to  
129 this equation:  $\sum^{n=6} = xi/xmax$  where for each contaminant and each year,  $xi$  = annual concentrations of  
130 considered stations and  $xmax$  = annual concentration maximum, a higher rank (i.e. 1) was accorded to the  
131 highest concentration value of PCBs, PAHs and metals for both 2015 and 2016 (Pain-Devin et al., 2014). Then, a  
132 global index was obtained by adding rank values for each condition (i.e. contaminant / year).  
133 In this context, during the present study, 9 sampling stations were followed in order to investigate different areas  
134 of interest along the gradients of contamination (Fig. 1): two stations in the south of the bay (i.e. Longues-sur-  
135 mer and Ver-sur-mer); two close to the mouth of the Seine (i.e. stations of Trouville-sur-mer and Cap de la  
136 Hève, in the south and north respectively); five in the north of the bay, downstream (i.e. stations of Octeville-sur-  
137 mer and Southern Antifer) and upstream from the Antifer Harbor (i.e. stations of Northern Antifer, Yport and  
138 Senneville-sur-mer).

139

#### 140 2.2. Procedure of prawn sampling

141 Adult specimens of *P. serratus* were collected on the intertidal rocky shore of the littoral of the Seine Bay using  
142 a hand-net. Water temperature, salinity, pH and dissolved oxygen were systematically recorded. Sexually mature  
143 male prawns were immediately shortlisted by the presence of secondary sexual characteristics, as described in  
144 Erraud et al. (2017). Prawns were brought to the laboratory in one hour, in 30 L-plastic containers supplied with  
145 the natural seawater of the sampling station, under oxygenation. Males were kept in the plastic containers under  
146 oxygenation until the analysis of DNA damage from 10 individual sperm suspensions (i.e.  $n = 10$  different  
147 specimens), the next morning.

148

#### 149 2.3. Procedure of the sperm DNA damage analysis

150 The measurement of DNA damage in the prawns' spermatozoa was performed according to the methodology  
151 described in Erraud et al. (2017). Beforehand, male specimens were weighed and the total cephalothoracic length  
152 (LCT) was measured. A fragment of uropod was removed and observed under a photonic microscope (i.e.  
153 EVOS® FL Auto Cell Imaging System; x100 and then x400) in order to determine the molt stage on the basis of  
154 the integumental morphogenesis according to the classification of Drach (1944) (Fig. 2). Spermatophores were



155 extracted by a gentle pressure between the fifth pair of pereopods, allowing the expulsion of spermatophores  
156 from the terminal ampullae. Then, spermatophores were transferred into 1.5 mL-microtube, weighed, immersed  
157 with 600  $\mu\text{L}$  of artificial seawater (adjusted to the hemolymphatic osmolality of *P. serratus* at i.e. 950 – 1000  
158  $\text{mOsmol.kg}^{-1}$ ) and ripped by pipetting up and down until the entire laceration of spermatophores. For each  
159 specimen tested in the present study, the mortality of sperm-suspension was assessed by a membrane  
160 permeability test after mixing cells 1:1(v/v) with an isotonic Trypan-blue dye solution (i.e. 0.4 % w/v). Cellular  
161 mortality was performed in KOVA® slides, using a photonic microscope (x400). Mortality measurements were  
162 performed on the spermatozoa suspensions of 20 males. To avoid the measurement of DNA damage due to  
163 cytotoxic events leading to an over-estimation, a minimum of ten different males displaying a mortality rate  
164 (< 15%) were selected for the assessment of DNA damage using the Comet assay ( $n = 10$ ).

165 Prior to the Comet assay, superfrosted microscope slides were first covered with a normal melting agarose-type I  
166 (0.8 % w/v) in  $\text{Ca}^{2+}$  -  $\text{Mg}^{2+}$  free PBS (i.e. 10 mM, pH 7.4) and dried overnight at ambient temperature. After  
167 collection of spermatozoa, 60  $\mu\text{L}$  of the cell suspension (i.e.  $10^6$   $\text{cells.mL}^{-1}$ ) were equally mixed with 60  $\mu\text{L}$  of  
168 1 % low-melting-point agarose-type VII in  $\text{Ca}^{2+}$  -  $\text{Mg}^{2+}$  free PBS (i.e. 10 mM, pH 7.4, 37°C - 0.5% final agarose  
169 concentration), and immediately deposited onto the coated slides and finally covered with a 24  $\times$  60 mm  
170 coverslip. From this point, all steps of the Comet assay protocol were performed in darkness with inactinic light  
171 to prevent additional DNA damage. Slides were cooled for 10 min at 4°C on ice for solidification of agarose.  
172 After removal of the coverslip, slides were placed in a freshly prepared lysing solution (i.e. 2.5 M NaCl, 100 mM  
173  $\text{Na}_2\text{EDTA}$ , 10 mM Tris, 1 % Triton X-100 and 10 % DMSO added immediately before use, pH 10) at 4°C in the  
174 dark for 1 hour. After cell lysis, slides were gently placed in a horizontal electrophoresis chamber filled with  
175 freshly prepared alkaline solution (i.e. 300 mM NaOH, 1 mM  $\text{Na}_2\text{EDTA}$ , pH > 13). DNA was then allowed to  
176 unwind for 15 min. Electrophoresis of DNA was performed in a standard Comet Assay Tank (i.e. 20 slides; 1050  
177 mL of alkaline solutions; 24 cm x 27 cm x 7 cm (W x L x H)) under  $0.83 \text{ V.cm}^{-1}$  for 24 min. After  
178 electrophoresis, slides were washed in a neutralization buffer (i.e. 0.4 M Tris-HCl, pH 7.5) at 4°C for 20 min.  
179 The last step consisted of a dehydration of slides in absolute ethanol for 15 min. DNA embedded in the slide was  
180 stained with 30  $\mu\text{L}$  of a DAPI solution at a concentration of  $2 \mu\text{g.mL}^{-1}$ . Slides were blindly observed using an  
181 epifluorescence-reversed microscope (Eclipse TE2000-U, Nikon®). Slide scoring was assessed by a visual  
182 scoring according to the method of Collins (2004) which was demonstrated as trustworthy as image analysis  
183 (e.g. Azqueta et al., 2011), and proved to be appropriate to detect DNA damage in sperm from *P.serratus*  
184 (Erraud et al. 2017). A minimum of 150 spermatozoa per slide was counted and classified into five categories of

185 Comet according to the degree of DNA damage, from 0 (i.e. no tail) to 4 (i.e. almost all DNA in the tail  
186 indicating highly damaged DNA). The DNA damage level was expressed in arbitrary units (i.e. AU) (Fig. 3).  
187 This arbitrary unit, graduated from 0 to 400, was calculated by applying the following formula: (percentage of  
188 nucleoids in class 0 x 0) + (percentage of nucleoids in class 1 x 1) + (percentage of nucleoids in class 2 x 2) +  
189 (percentage of nucleoids in class 3 x 3) + (percentage of nucleoids in class 4 x 4)

#### 190 *2.4. Characterization of a DNA damage baseline level in prawns from a reference station*

191 In order to describe the natural variability of DNA damage in spermatozoa of *P. serratus* and to define a baseline  
192 level, prawns were sampled monthly at the station at Yport, from May to November during two years: 2015 and  
193 2016 (see section 2.1). Yport, which is located north of Antifer harbor (Fig. 1), is admitted to be a slightly  
194 polluted station according to ROCCH data records (see section 2.1) and is used as a reference station in our  
195 laboratory (e.g. Rioult et al., 2014). The period from May to November corresponds to the period during which  
196 *P. serratus* specimens are found on the rocky shore of the intertidal zone until they return to greater depths  
197 (Campillo, 1975). Prawns were considered and analyzed as previously described (see section 2.3).

#### 198 *2.5. Biomonitoring of natural populations along the Seine Bay coastline*

199 All stations previously described (see section 2.1; Fig. 1) were monitored during the autumns of 2015 and 2016  
200 between mid-September and mid-October, except for Longues-sur-mer and Northern Antifer, which were added  
201 during the campaign of 2016. All of these sampling sites exhibited different levels of anthropogenic pressure  
202 according to ROCCH observations. Prawns were sampled in the intertidal zone (see section 2.2), except for the  
203 stations at Ver-sur-mer and Longues-sur-mer, where prawns were caught by a fisherman using prawn traps close  
204 to the coast. Prawns were considered and analysed as previously described (see sections 2.2 and 2.3).

#### 206 *2.6. Statistical analysis*

207 Statistical analyses were performed with the R studio software v0.99.903 (RStudio Inc.). For values of DNA  
208 damage for Yport, normality and homoscedasticity were assessed by a Shapiro-Wilk test and a Bartlett test  
209 respectively. Inter-individual variability of DNA damage for each month was then assessed using ANOVA test.  
210 The influences of temperature, total weight and total cephalothoracic length on the natural variability of DNA  
211 damages were assessed using mixed effect models including sampling date as random effect to take into account  
212 pseudo-replication in the dataset. The relationship between DNA damage and the molt stage was assessed using  
213 an ANOVA test. A maximum reference threshold for DNA damage of monthly means was defined on the basis

214 of the variation in the mean of values measured on 10 prawns simultaneously collected from the reference station  
215 at a common date (95<sup>th</sup> percentile). This threshold value of DNA damage was used thereafter for the passive  
216 monitoring experiment in order to test whether the mean of recorded values (also 10 prawns per site) conform to  
217 this reference distribution. A comparison was also made between the two years at each site using an ANOVA  
218 test followed by a post hoc test Tukey HSD.

219

### 220 3. Results

#### 221 3.1. The range of natural variability of spermatozoa DNA damage and definition of a reference distribution

222 Figure 4 shows the level of DNA damage measured in the spermatozoa of Yport's prawn population (i.e.  
223 reference population) from May to November in 2015 and May to October 2016. In November 2016, prawns had  
224 already migrated from the intertidal zone. Water temperature, salinity, pH and dissolved oxygen were recorded  
225 monthly throughout the sampling campaigns. No variability of pH (i.e.  $8.21 \pm 0.9$ , mean  $\pm$  SD), salinity (i.e. 33-  
226 35 PSU) and dissolved oxygen (i.e.  $8.09 \pm 0.24$  ppm, mean  $\pm$  SD) was observed during the monitoring. The  
227 temperature was the only registered physicochemical factor displaying a seasonal cycle with remarkable  
228 variations ranging from 11.8 to 19.2 °C.

229 DNA damage was measured for 7 months in 2015 and again for 6 months in 2016 (10 prawns per sampling  
230 date). DNA damage values ( $n = 130$  prawns) were normally distributed (Shapiro-Wilk test,  $p = 0.0632$ ) and  
231 homoscedasticity among sampling dates was verified (Bartlett test,  $p = 0.0923$ ). A weak but significant  
232 variability of DNA damage levels was detected between sampling dates (ANOVA test,  $p = 0.0206$ ). However,  
233 the DNA damage level of spermatozoa measured in prawns from Yport presented no variability trends. Potential  
234 relationships between DNA damage and temperature or intrinsic factors were thus investigated. Figure 5A shows  
235 the relationship between the average level of DNA damage in spermatozoa of the 10 prawns of each month and  
236 the temperature recorded monthly in the field. No correlation has been observed (Pearson's correlation test,  $p =$   
237  $0.2926$ ;  $r = 0.09$ ). The weight and the total cephalothoracic length recorded during the sampling of prawns from  
238 the intertidal zone ranged from 7 to 33 mm and from 420 to 2380 mg, respectively. Figure 5BC represents the  
239 relationship between DNA damage of 130 prawns and their weight or total cephalothoracic length. No  
240 significant influence of these biotic factors with DNA damage level has been observed in mixed effect models ( $p$   
241  $= 0.2644$  for the cephalothoracic length;  $p = 0.4276$  for the weight). Finally, the relationship between the molt  
242 stages and DNA damage of spermatozoa obtained on the 130 prawns was assessed (Fig. 6). Sampled prawns  
243 were found in all molting stages with a frequency of 2.6 % in stage A, 28.2 % in stage B, 19.6 % in stage C, 20.5

244 % in stage D0, 12.8 % in stage D1', 4,3 % in stage D1'', 7.7 % in stage D1''' and 4.3 % in stage D2. No  
245 significant difference was obtained (ANOVA test,  $p = 0.321$ ). Hence, based on this chronological data set, a  
246 reference distribution was established. The mean baseline level was established at  $52.6 \pm 5.6$  A.U and the upper  
247 95% unilateral confidence threshold was 61.7 A.U. This maximum damage threshold is based on the variation  
248 of the monthly mean of DNA damage levels ( $n = 10$  prawns), which notably presented a quite small variability  
249 over all months (Variation Coefficient - VC = 10.6 %). All the mean monthly values of DNA damage recorded  
250 during the two-year monitoring in Yport fall below the defined 95% unilateral confidence threshold (Fig. 4).

251  
252 *3.2. Measure of DNA damage in sperm from prawns sampled in different populations along the Seine Bay*  
253 The environmental factors were in the previously observed range of the monthly sampling from May to  
254 November 2015-16 in Yport (i.e. temperature: 13.8 - 19.2 °C; Salinity: 33 - 35; pH:  $8.17 \pm 0.5$  and dissolved O<sub>2</sub>:  
255  $7.95 \pm 0.15$  ppm). The cephalothoracic size and total weight of prawns sampled along the rocky shore using a  
256 hand net (see section 2.1) ranged from 13 to 33 mm and 525 to 2197 mg, respectively. In contrast, prawns  
257 sampled by the fisherman at the stations of Ver-sur-mer and Longues-sur-mer displayed slightly bigger size and  
258 higher weight (i.e. total weight: 1672 - 2851 mg and LCT: 26 - 34 mm). Figure 7 presents the passive  
259 biomonitoring performed on 7 sites in October 2015 and 9 sites in October 2016 on the Seine Bay to assess the  
260 level of DNA damage. Levels of DNA damage displayed no significant variation between 2015 and 2016 in the  
261 different sites (HSD Tukey,  $p > 0.05$ ). All the sites situated north of the Antifer seawall and south of the Seine  
262 Estuary presented mean DNA damage levels below the maximum damage threshold of 61.7 AU defined in the  
263 reference population (see section 3.1). In contrast, the highest levels of DNA damage were observed in the north  
264 of the Seine Bay at the stations of Southern Antifer, Octeville-sur-mer and Cap de la Hève, displaying a  
265 maximum of  $90.2 \pm 13.8$  A.U,  $81.0 \pm 13.3$  A.U and  $85.0 \pm 15.8$  A.U, respectively. A significant decrease of 54  
266 % of the mean level of DNA damage was observed in 2016 between the south and the north of the seawall of  
267 Antifer (i.e.  $90.2 \pm 13.8$  A.U and  $58.4 \pm 27.0$  A.U respectively) (ANOVA,  $p = 0.0327$ ). However, it can be  
268 underlined that the prawns of the northern station showed a significant inter-individual variability  
269 (VC = 46.2 %).

#### 271 4. Discussion

272 Initially, biomarkers were developed in ecotoxicology to assess environmental quality (McCarthy & Shugart,  
273 1990). Even so, they are still rarely used in biomonitoring programs especially in spatial and temporal large-

274 scale studies due to the lack of control of their natural variability. This natural variability caused by  
275 environmental or intrinsic biotic factors other than contaminants can represent a real problem in the  
276 identification of the effects of contamination. The only alternative to improve biomarker interpretation is to  
277 understand both the natural inter-individual variability, the seasonal variability and the influence of other  
278 potential confounding factors from populations of reference sites, in order to establish reference values taking  
279 into account this variability (Coulaud et al., 2011; Jemec et al., 2010; Xuereb et al. 2009). When the analysis of  
280 the chronological and/or spatial data set displays a non-significant variability for a biomarker under parameters  
281 other than the contaminant, a baseline can be constructed directly. However, many biomarkers have shown a  
282 marked seasonal variability (Dixon et al., 2002; Jha, 2008; Sanchez et al., 2008; Wiklund and Sundelin, 2004;  
283 Barrick et al., 2016). If the results are predominantly affected, the origins of the variability have to be identified  
284 and integrated to minimize the risk of misinterpretation. (Wirzinger et al., 2007). The variability can then be  
285 minimized by sampling methodology optimizations such as the selection of a standard range of organisms  
286 (Xuereb et al. 2009) or about sampling periods (Akcha et al. 2004). In another way, when trends clearly appear,  
287 the variability can be directly lessened during the data analysis by using mathematic models (Coulaud et al.  
288 2011; Barrick et al., 2016). Conversely, if the biological response shows stochastic fluctuations, it consequently  
289 has no potential to be used in the field (Jubeaux et al., 2012).

290 The aim of the present study was the assessment of the natural variability of DNA damage on *P. serratus*  
291 spermatozoa to propose a reference value distribution allowing the use of this response as biomarkers of  
292 genotoxicity in the field. The final step of this process aimed to assess the discriminant power of the biomarker  
293 throughout regionally contrasting contaminated stations.

294

#### 295 4.1. Characterization of the natural variability and definition of a baseline level

296 In the present study, the inter-individual and seasonal variability of the sperm DNA damage basal level was  
297 assessed during a two-year period (from May to November in 2015 and 2016) within a reference wild  
298 population of *P. serratus*, Yport. Prawns displayed relatively low damage levels (i.e. 53 A.U. in mean). A  
299 residual but significant variation (i.e. VC of the monthly means = 11 %) of the response has been characterized  
300 at the outcome of the data analysis. Our results are consistent with the ones reported by Lacaze et al. (2011b) in  
301 spermatozoa of the freshwater shrimp *Gammarus fossarum*. Their work showed no difference of sperm DNA  
302 damage levels in gammarids collected monthly in two reference stations from January to December 2009 and  
303 2010. Conversely, other author groups have reported considerable spatio-temporal variations of DNA damage

304 levels in different cellular types (i.e. hemolymph cells, and gill cells) in molluscs such as *Mytilus sp* and fish  
305 such as *Limanda limanda* (Akcha et al., 2004; Dévier et al., 2013; Frenzilli et al., 2001; Magni et al., 2006;  
306 Pisanelli et al., 2009; Shaw et al., 2000).

307 The diagnosis of a residual variation led us to examine the influence of different environmental or intrinsic  
308 biotic factors in order to identify potential tendencies which could be diminished by mathematical models or  
309 avoided by refining the sampling strategy. Contrary to the continental and estuarine environment, coastal and  
310 marine areas appear much buffered in terms of salinity and pH. In the scale of the Seine Bay, variations of  
311 these parameters are mostly induced in the winter, where desalinated water provided by the Seine flood is  
312 tackled to the coast, decreasing the salinity slightly to a level of 30 in the internal area between the Cap line of  
313 Antifer and Ouistreham and all the coasts of the Bay to less than 33. However, the period of coastal presence of  
314 prawns (i.e. from May to November) coincides with the period of stability of these parameters in the Seine Bay.  
315 On the local scale of a rocky shore, it may have variations in relation to the tidal rhythm (i.e. desiccation and  
316 fresh water resurgence). However, during our monitoring, the water temperature was the only measured  
317 environmental factor which displayed remarkable seasonal fluctuation (i.e. from 11.8 to 19.2 °C). In spite of this  
318 contrast, the statistical analysis showed no significant correlation between the water temperature and the  
319 measured DNA damage in prawn spermatozoa. Several studies have however demonstrated that the extremities  
320 of environmental temperature gradient seem to affect the DNA integrity in different taxa and cell types. Indeed,  
321 an increase of the baseline level of DNA damage was observed in erythrocytes of the dab *Limanda limanda*  
322 (Akcha et al. 2004), in cells from the gills, kidney, liver and blood of the mullet *Liza aurata* (Oliveira et al.,  
323 2010) and in haemocytes of the mussel *Mytilus galloprovincialis* (Pisanelli et al. 2009) during the coldest  
324 months of the year. An inverse tendency was also reported for hot summer temperatures such as in the digestive  
325 gland cells and gill cells of *Mytilus sp* (Shaw et al. 2000; Magni et al. 2006) or in gill cells of *Dreissena*  
326 *polymorpha* (Michel et al., 2013) and in blood cells of two species of fish, the gray mullet and the sea catfish  
327 (De Andrade et al., 2004). Conversely, Lacaze et al. (2011b) recorded no effect of water temperature on DNA  
328 integrity of spermatozoa of Gammarids during a laboratory-controlled experimentation (i.e. from 6 to 24 °C) as  
329 well as in the field (i.e. from 5 to 19 °C) except for the month of July in their reference river where a significant  
330 increase in DNA damage was measured. In this last case, this increase was explained by a global stress with a  
331 temperature exceeding the optimal living temperature (i.e. > 24°C for 3 days) and the minimal flow rates which  
332 could be associated with a decrease of the oxygen concentration.

333 Among the intrinsic biotic factors capable of influencing the baseline level of DNA damage, the age or the life  
334 stage was already highlighted in previous works. For example, Akcha et al. (2003) demonstrated that DNA  
335 damage measured in blood cells of adult dabs was higher than that in juveniles. A positive correlation between  
336 the DNA damage in blood cells and the size was observed in the three-spined stickleback *Gasterosteus*  
337 *aculeatus* (Wirzinger et al., 2007). Nevertheless, these authors did not exclude a possible effect of chemical  
338 stress due to the contamination in their reference station. That has been confirmed by Santos et al. (2014), who  
339 demonstrated in the same biological model that there is no correlation between fish length and the level of DNA  
340 damage in a reference site. The positive influence of age on DNA damage was also observed in the gill cells and  
341 haemocytes of blue mussels (Rank et al., 2005). The authors explained this difference by a higher  
342 biotransformation capacity of adults in comparison with juveniles. During the present study, the analysis of the  
343 distribution of the prawn cephalothorax size on the basis of work by Campillo (1975) (i.e. concerning the  
344 description of cohorts within *P. serratus* populations of Roscoff, a station located on the French Coast of the  
345 English Chanel; 1975), showed that more than 94 % of sampled specimens corresponded to the age class 0  
346 (LCT < 27 mm), while only 6 % seem to belong to the age class 1 (27 < LCT < 36 mm) and none represented  
347 the age class 2 (LCT ≥ 36 mm). This estimation suggests that the employed capture procedure (i.e. manual  
348 capture using a hand-net on intertidal rocky shore) led to a homogenous sampling in terms of the age of prawns.  
349 Despite the lack of variability in terms of age, the high level of disparity in terms of size and weight in the age  
350 class 0 made us wonder about the potential interaction between the DNA damage in spermatozoa and these two  
351 intrinsic biotic parameters. Our results show no significant correlation for total cephalothoracic length and total  
352 weights ranging from 7 to 33 mm and from 420 to 2380 mg, respectively.

353 In palaemonids, as for most decapods, sperm resides in the male seminal vesicle in the form of spermatophore  
354 (i.e. a medial mucus mass encapsulated in a non-cellular envelope). This particularity offers the advantage to  
355 collect and to work only on homogeneous mature spermatozoa populations and therefore to avoid a potential  
356 difference in the sensitivity between the different stages of spermatogenesis depending if cells have been  
357 exposed during their differentiation or in a mature stage (i.e. as suggested in *G. fossarum* by Lacaze et al.,  
358 2011b). However, males being continuously ready to mate, even outside of the reproduction periods (i.e. from  
359 November to May in *P. serratus*; according to Campillo, 1975), the issue of the aging of spermatozoa contained  
360 in the spermatophores can legitimately be addressed. Parnes et al. (2006) reported the first evidence that the  
361 reproductive cycle of male prawns is strictly associated with their molt cycle in *Litopenaeus vannamei*, intact  
362 spermatophores disappearing about 12 h premolt and a new pair appearing in the ejaculatory bulbs the day after

363 exuviation. Consequently, the different molt stages were used here as chronological markers to assess the  
364 influence of retention time of spermatozoa in the spermatophore on their DNA integrity. No significant effect  
365 was observed. On the basis of the work of Richard (1978) relative to molt cycle duration in *P. serratus* and  
366 considering prawns sampled in this present work, these results suggested that the spermatozoa could be kept  
367 more than one month in the spermatophores without any DNA degradation.

368 Ultimately, a low residual variability of the sperm baseline level of DNA damage was observed, which does not  
369 seem to be explained by the environmental or intrinsic factors identified as the most important. Consequently, a  
370 reference value was generated from the Yport chronological data set with a mean value of DNA damage of  $52.6$   
371  $\pm 5.6$  A.U and a maximum threshold corresponding to the unilateral 95% confidence intervals of 61.7 A.U from  
372 which a superior DNA damage level could be interpreted as a modulation, resulting from an exposure to  
373 contaminants. However, this methodology was developed from only one reference station on prawns of the age  
374 class 0 and on the water temperature conditions of spring and summer (i.e. from 11.8 to 19.2 °C). As  
375 recommended by OSPAR Convention (2013), further studies should investigate several reference stations on a  
376 larger range of physicochemical parameters in order to consolidate the robustness of this reference distribution in  
377 view to a large-scale deployment.

378  
379 *4.2 Relevance of DNA damage in sperm of prawns in the spatial discrimination of the genotoxicity in different*  
380 *sites of the Seine Bay*

381 The Seine Bay is considered to be one of those most affected by trace metals, PAHs and polychlorinated  
382 biphenyls (PCBs) in Europe, presenting a well-defined gradient of contamination which reduces from the mouth  
383 of the estuary towards the extremities of the Bay. In order to assess the relevance of the use of a reference  
384 distribution presently proposed, different stations were investigated along the Seine Bay coastline. Globally, the  
385 results of this work revealed contrasting genotoxic impregnation levels coherent with the contamination gradient  
386 of the Seine Bay (i.e. on the basis of mussel bioaccumulation measurements; Tab. 1).

387 Considering the slightly contaminated stations located at the extremity of the bay (i.e. Senneville-sur-Fécamp in  
388 the north, and Longues-sur-mer and Ver-sur-mer in the south), the levels of sperm DNA damage were below the  
389 maximum threshold (i.e. previously defined by the data set recorded at the station of Yport). These results  
390 demonstrate in hindsight the spatial robustness of this reference distribution. Otherwise, the incidence of age  
391 class of prawns on levels of sperm DNA damage may be questioned in our study, since the prawns from Ver-sur-  
392 mer and Longues-sur-mer sampled in deeper water by a fisherman, displayed a frequency of age class 1 (i.e. 90



393 % and 100 % on average, respectively) that was higher than those observed during the samplings performed at  
394 Yport (i.e. 6 % on average). The results tend to confirm that the age class of prawns has no incidence on sperm  
395 DNA integrity. They suggest also that different sampling methods (i.e. hand net and prawn trap) can be used to  
396 monitor this biomarker in Palaemonids prawns.

397 Regarding the stations under the influence of the Seine plume, all the stations in the north between the mouth of  
398 the estuary and the Antifer Seawall (i.e. Cap de la Hève, Octeville-sur-mer; Southern Antifer) presented  
399 significant levels of DNA damage ranging from 31 to 46 % above the maximum threshold. Remarkably, a  
400 drastic decrease of DNA damage from the southern to the northern Antifer Seawall was observed despite a  
401 spacing of only 1.2 kilometers, highlighting the pronounced effect of the seawall on the drift of the chemical  
402 toxic load associated to the Seine plume. In the south, Trouville-sur-mer displayed a surprisingly low level of  
403 DNA damage in clear opposition to the stations placed near the mouth of the Seine estuary in the north (i.e. Cap  
404 de la Hève). It can be noted for this station that no significant DNA damage level was reported, despite a strong  
405 contamination index recorded in the ROCCH station of Villerville (i.e. stations only spaced 6.5 Km apart). It can  
406 be rationally hypothesized that, in spite of the proximity of these two stations, Trouville-sur-mer has been less  
407 exposed to the Seine plume (i.e. transported along the northern coastline by the longshore drift) than Villerville,  
408 which is located nearest the estuary mouth (Fig 1). In the same way, similar genotoxic impacts were observed  
409 from Cap de la Hève to Southern Antifer in spite of a halving of the contamination index between these two  
410 stations. This gap could be explained by the difference of the contaminant bioavailable fraction between mussels  
411 and prawns or by the fact that the most conventional contaminants reported in the survey network do not explain  
412 all the genotoxic impact. This report shows the interest of biological monitoring, highlighting, thanks to the  
413 threshold value, a chemical pressure not identified by the ROCCH observations.

414 Finally, the proposed methodology seems to be relevant to diagnose finely and robustly abnormal levels of  
415 sperm DNA damage in *P. serratus* and consequently to characterize the genotoxic quality of water bodies along  
416 the shoreline. However, it could be interesting to improve the ecological relevance of this tool to study the  
417 impact of sperm DNA damage on parameters of reproductive and recruitment success (e.g. fertilization and  
418 hatching success; abnormality rate during the early and late phases of embryo-larval development). So, the  
419 abnormal sperm DNA damage could be interpreted relative to the potential risk incurred for the populations. In  
420 this way, several authors have demonstrated, in laboratory *in vivo* and *ex vivo* exposures of semen, a significant  
421 relationship between sperm DNA damage and progeny defects in fish species (Devaux et al. 2011, Santos et al.  
422 2013) as well as in invertebrate species (Lacaze et al. 2011b; Lewis & Galloway, 2009). More recently, Devaux

423 et al. (2015) have reported such relationships in the fish nase (i.e. *Chondrostoma nasus*) sampled in a heavily  
424 contaminated station, suggesting that levels of DNA damage sufficient to lead to a fitness impairment could be  
425 found in a realistic environmental scenario. However, a transversal reading of the whole of these works tends to  
426 show that the level of sperm DNA damage required to cause significant alterations of reproductive success are  
427 dependent on the studied species and/or the considered endpoint. Consequently, it is difficult to replace the high  
428 levels of sperm DNA damage measured during the present study within the Southern Antifer, Octeville and Cap  
429 de la Hève populations with respect to the reproduction and progeny risks for prawns, simply on the basis of the  
430 literature analysis. Future work is thus needed to investigate this link in palaemonid prawns.

431

## 432 5. Conclusion

433 The aim of this study was to assess the natural variability of DNA damage on *P. serratus* spermatozoa in order to  
434 determine a baseline level. The results showed that DNA damage on spermatozoa of *P. serratus* are not related  
435 to intrinsic biotic factors (i.e. total size; total weight; molting stage) and to environmental factors (i.e. the water  
436 temperature) obtained with monthly sampling in 2015-2016 at Yport. We have proposed a baseline level and  
437 maximum threshold beyond which the increase of DNA damage may be attributed to present or past  
438 contamination exposure and so significant differences between sites could be easily reported. Finally, the  
439 biological data and the reference distribution appear to make it possible to discriminate between the sites  
440 according to the pressure/contamination gradient of the Seine Bay.

441

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446

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625 surveys. *Aquat. Toxicol.* 93, 225–233.

## 628 Legends

629 Table 1. Annual means (n = 2 analyses per year) of cumulated concentrations of 12 PAHs, 9 PCBs and 7 metals  
630 measured in mussel soft-tissues (*Mytilus edulis*) from six sampling sites (i.e. Yport; Southern Antifer; Cap de la  
631 Hève; Villerville; Ouistreham and Port-en-Bessin). These data were generated by the French marine  
632 environment monitoring network (ROCCH; “<http://www.ifremer.fr/envlit/>”). PCBs and PAHs concentrations are  
633 expressed in µg/kg-dry weight and metals concentrations in mg/kg-dry weight. A contamination index was  
634 proposed for each station on the basis of a rank transformation (see section 2.4)

636  
 637 Figure 1. Localization of the sampling stations along the Seine Bay (Normandy; France). White point surround  
 638 of black = stations of biomonitoring without ROCCH data; white point surround of red = stations of  
 639 biomonitoring with ROCCH data; red point surrounded of black = ROCCH data without sampling.

640  
 641 Figure 2. Observation of the setogenesis morphological changes during the molt cycle from A to D2 (x 100 and  
 642 x 400) : Stage A (early postmolt): the setal lumen filled with setal matrix; Stage B (late post molt): retraction of  
 643 the setal matrix and the beginning to internal cone formation; Stage C (intermolt): empty setal lumen and internal  
 644 cone; Stage D0 (onset of premolt): separation of the cuticle and epidermis; Stage D1' (early premolt): formation  
 645 of a circular fold around the nervous cluster of setae; Stage D1'' (intermediate premolt): the circular fold  
 646 reaches its maximum depth beginning of secretion; Stage D1''' (late premolt): the walls of the fold secrete the  
 647 skeletal case; Stage D2 (late premolt): formation of the preexuvial wall of the future skeletal.

648  
 649 Figure 3. Visual classification of DAPI-stained Comet figures in *Palaemon serratus* spermatozoa (class 0 to 4)  
 650 adapted from Collins (2004): class 0, no damage; class 1, low damages; class 2, medium damages; class 3, high  
 651 damages; class 4, complete damage.

652  
 653 Figure 4. Natural variability of DNA damage (expressed in arbitrary units AU) measured in *Palaemon serratus*  
 654 spermatozoa during its period of attendance on the coast from May to November 2015-2016 at Yport (reference  
 655 site). Results are shown in boxplot (i.e. the median, the first and the third quartiles, the non-outliers range and the  
 656 outliers). The mean (red point) was added to boxplots to provide as much information as possible of data set.  
 657 White boxplots = 2015, grey boxplots = 2016; continuous line represents the bi-annual mean of DNA damage;  
 658 the dashed lines represent the 95% unilateral confidence intervals (n=10).  
 659

660  
 661 Figure 5. Relationship between the temperature (A), the total cephalothoracic length (B) or the total weight (C)  
 662 and DNA damage of spermatozoa from males *P. serratus* sampled in Yport from May to November 2015-2016  
 663 (n=130).  
 664

665  
 666 Figure 6. Relationship between the molting stage and DNA damage of spermatozoa from *P. serratus* sampled in  
 667 Yport from May to November 2015-2016 (n=130).  
 668

669  
 670 Figure 7. Sperm DNA damage measured in field populations of *Palaemon serratus* from different stations along  
 671 the Seine Bay sampled on 7-10 sites of the Seine estuary and the bay of Seine during autumn 2015-2016. (n=10)  
 672 Results are shown in boxplot (i.e. the median, the first and the third quartiles, the non-outliers range and the  
 673 outliers). The mean (red point) was added to boxplots to provide as much information as possible of data set.  
 674 White boxplots = 2015, grey boxplots = 2016; continuous line represents the bi-annual mean of DNA damage at  
 675 Yport; the dashed lines represent the 95% unilateral confidence intervals (n=10). Stations are presented from the  
 676 North to the South of the Seine bay and the Antifer seawall and the Seine estuary are replaced with vertical  
 dashed lines.

Table 1 :

Mussels sampling sites	PCBs		PAHs		Metals		Contamination index
	$\mu\text{g}/\text{kg-dry weight}$ 2015	2016	$\mu\text{g}/\text{kg-dry weight}$ 2015	2016	$\text{mg}/\text{kg-dry weight}$ 2015	2016	
Yport	137.2	/	93.7 (mean of 2013-2014)		72.7	102.9	1.8
Antifer south	293.7	357.7	67.6	233.0	176.9	140.5	3.2
Cap de la Hève	<b>455.9</b>	<b>541.9</b>	<b>373.4</b>	<b>620.3</b>	<b>337.8</b>	<b>174.5</b>	6
Villerville-sur-mer (Trouville-sur-mer)	401.9	488.0	195.8	213.0	293.3	132.1	4.3
Ouistreham (Ver-sur-mer)	166.6	189.1	137.5	121.4	242.43	86.4	2.4
Port-en-bessin (Longues-sur-mer)	75.7	77.2	78.4	65.7	259.7	112.4	2.0

Figure 1.

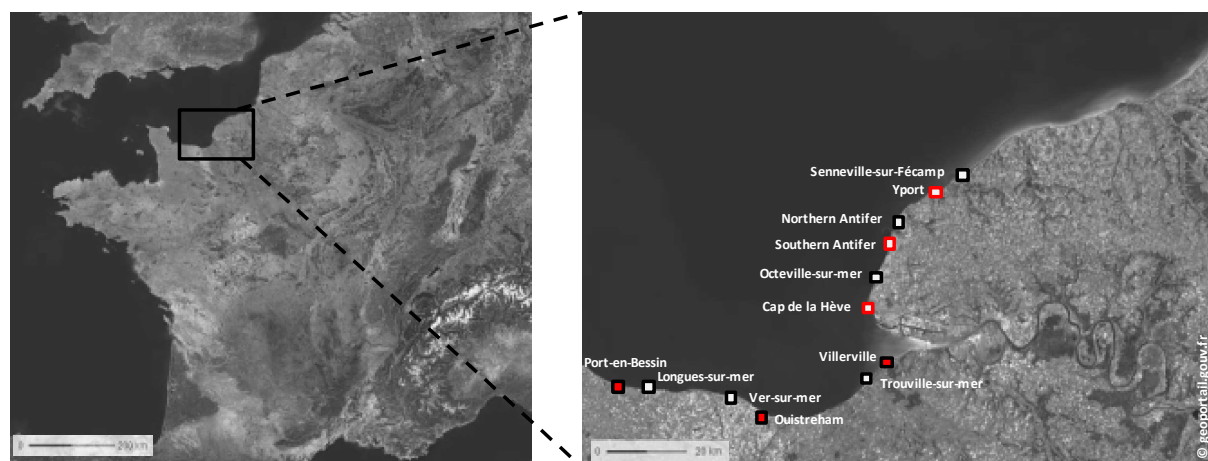




Figure 2.

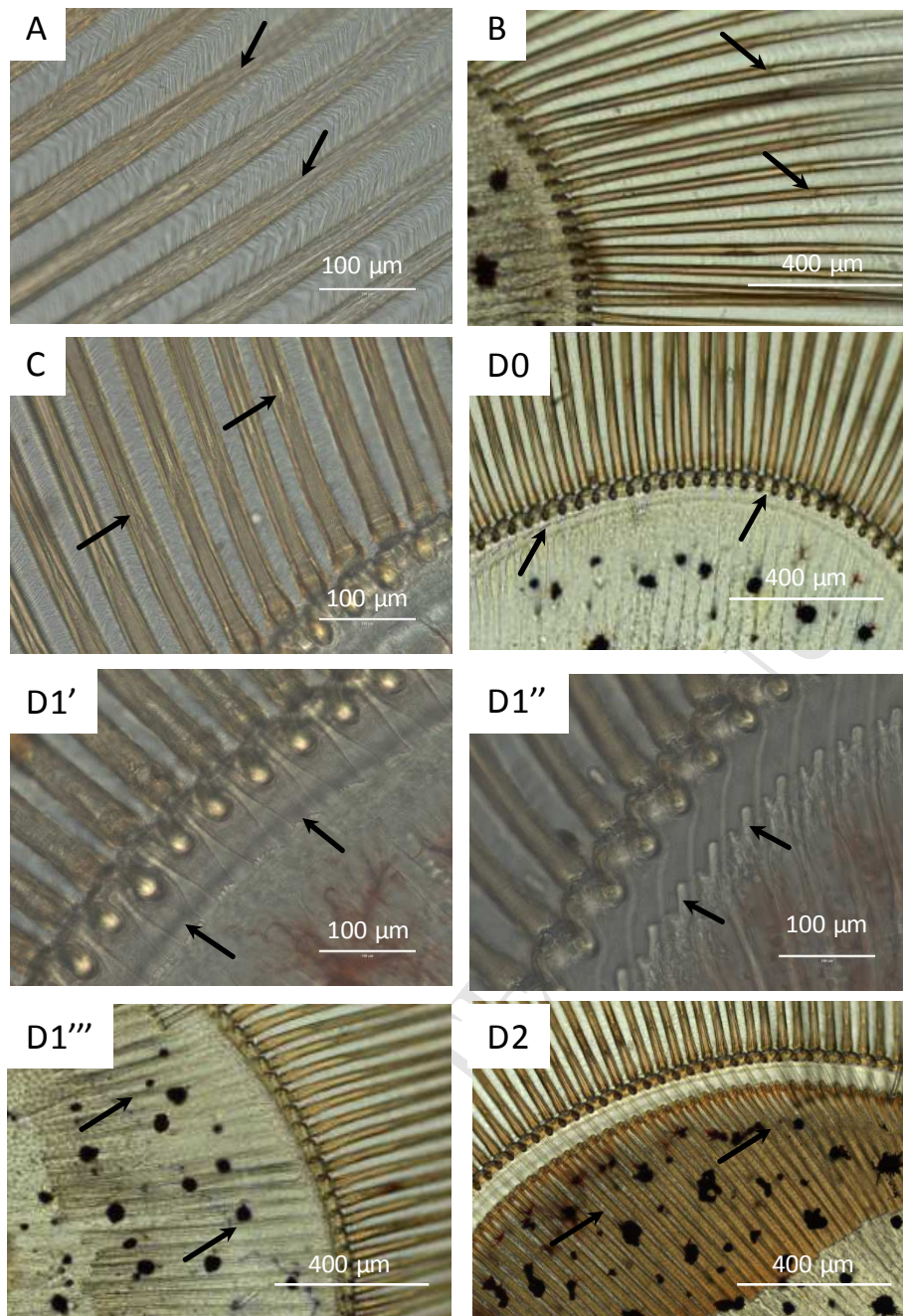


Figure 3

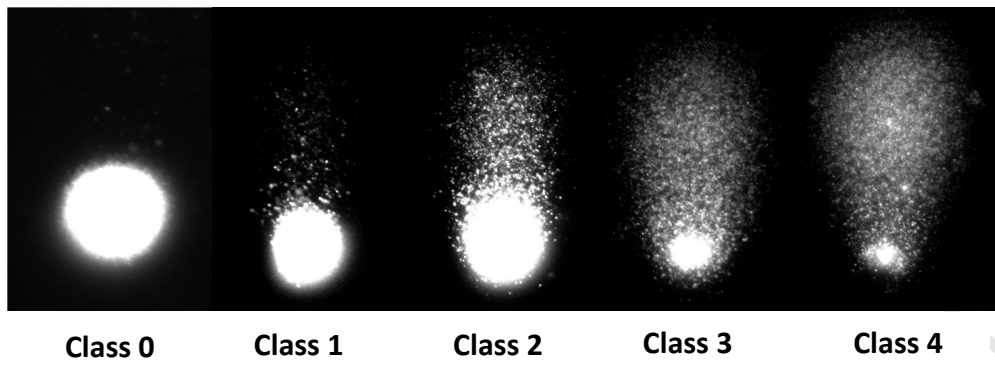


Figure 4.

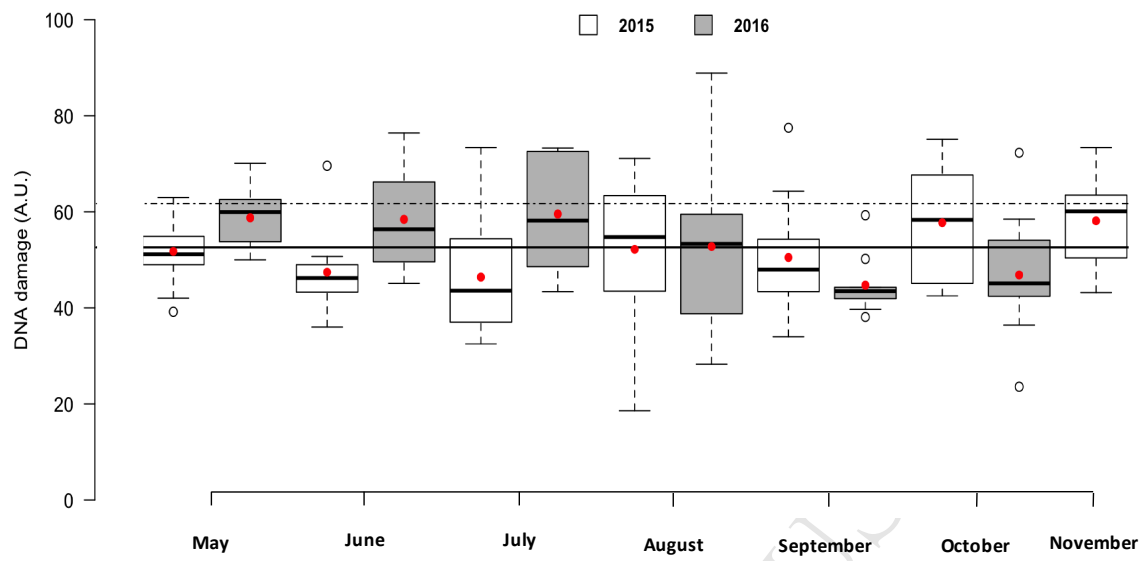


Figure 5.

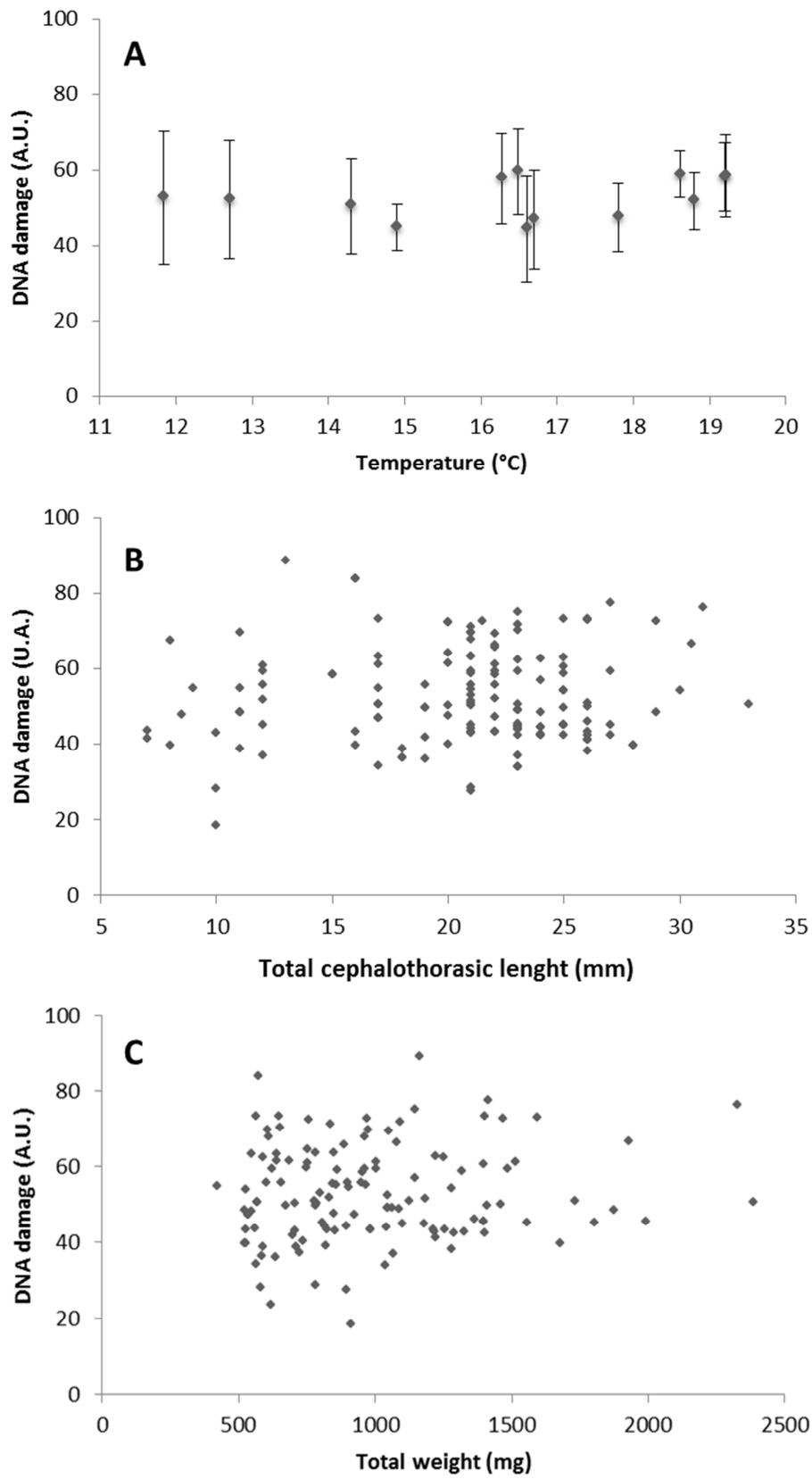


Figure 6.

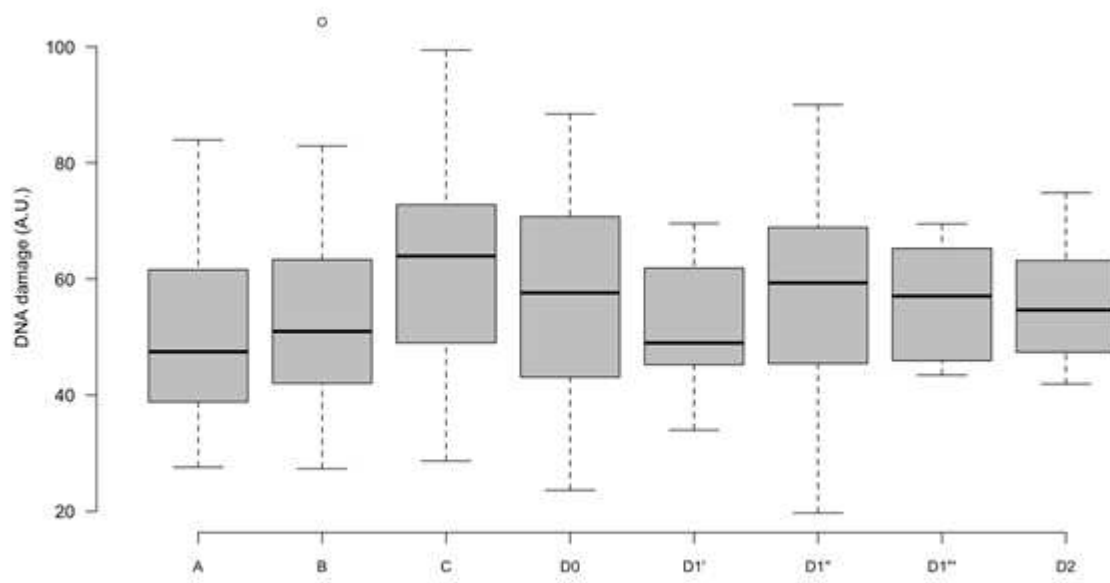
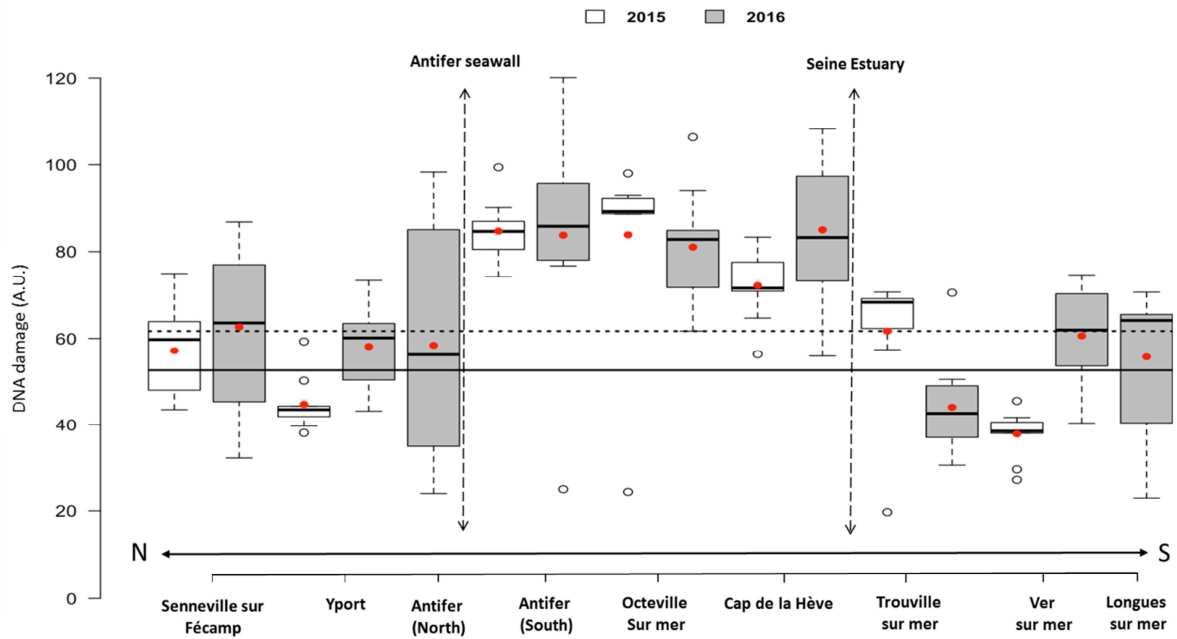


Figure 7.



**Highlights**

- We propose a reference distribution for sperm DNA integrity in *Palaemon serratus*
- The influence of main confounding factors on sperm DNA integrity was quantified
- Our reference distribution proved to be robust to environmental conditions
- Contamination impacts can be revealed by this biomarker to assess water quality

ACCEPTED MANUSCRIPT