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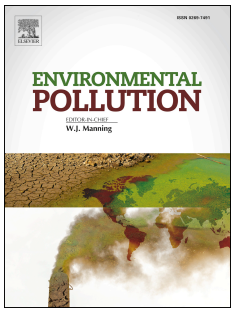
Accepted Manuscript



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PII: S0269-7491(18)33271-8

DOI: https://doi.org/10.1016/j.envpol.2018.11.006

Reference: ENPO 11833

To appear in: *Environmental Pollution*

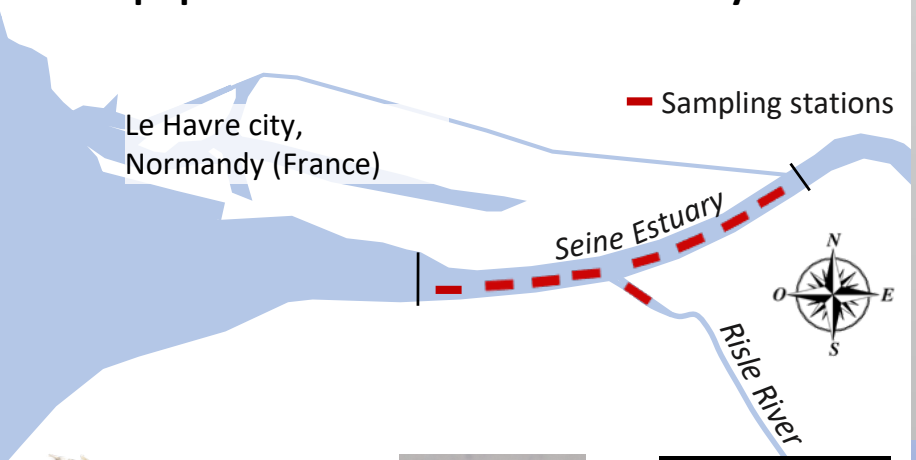
Received Date: 16 July 2018

Revised Date: 31 October 2018

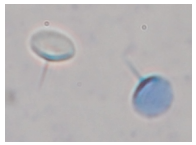
Accepted Date: 2 November 2018

Please cite this article as: Erraud, A., Bonnard, M., Geffard, O., Chaumot, A., Duflot, Auré., Geffard, A., Forget-Leray, Joë., Xuereb, B., Assessment of sperm DNA integrity within the *Palaemon longirostris* (H. Milne-Edwards, 1837) population of the Seine estuary, *Environmental Pollution* (2018), doi: https://doi.org/10.1016/j.envpol.2018.11.006.

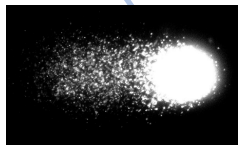
Sperm DNA integrity of *Palaemon longirostris* population from the Seine estuary



Palaemon longirostris



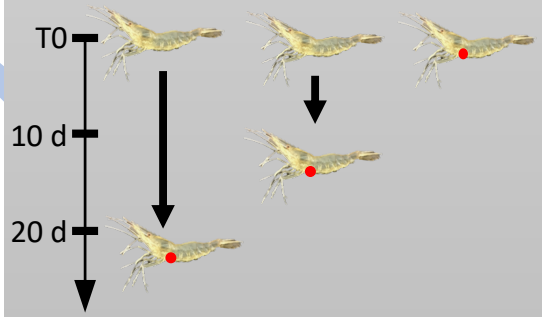
Sperm cell



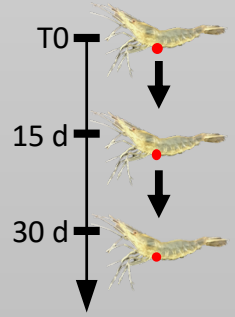
Comet assay

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)



➢ **Persistence of DNA damage**



✓ Decrease of sperm DNA damage after the first renewal

➢ **Definition of a baseline level**

● Extraction and Comet assay

2) Deployment in biomonitoring

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

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

Alexandre Erraud[1], Marc Bonnard[2], Olivier Geffard[3], Arnaud Chaumot[3], Aurélie Duflot[1], Alain Geffard[2], Joëlle Forget-Leray[1], Benoit Xuereb[1]*

[1] Normandie Univ, UNIHAVRE, UMR-I 02 SEBIO, FR CNRS 3730 SCALE, 76600 Le Havre, France

[2] Université Reims Champagne Ardenne, UMR-I 02 SEBIO, 51100 Reims, France.

[3] IRSTEA, UR RiverLY Laboratoire d'écotoxicologie, centre de Lyon-Villeurbanne, F-69625 Villeurbanne, France

* Corresponding author

Email: benoit.xuereb@univ-lehavre.fr

Tel: +33 2 32 85 99 11; fax: +33 2 32 74 45 05

Abstract

The interpretation of biomarkers *in natura* should be based on a referential of expected values in uncontaminated conditions. Nevertheless, to build a reference data set of biomarker responses in estuarine areas, which receive chronic pollution loads due to their transition position 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 define a baseline for the measurement of sperm DNA damage by Comet assay in the estuarine prawn *Palaemon longirostris.* For that, sperm 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) recovery of wild *P. longirostris* specimens from the Seine estuary, in laboratory conditions. Then, the levels of sperm DNA damage recorded within the *P. longirostris* population of the Seine estuary, during six campaigns of sampling from April 2015 to October 2017, have been interpreted according to the defined threshold values. The results showed a persistence in the level of DNA damage after 20-day in clean environment with the passive recovery. This strategy was inconclusive to reach a baseline level but it revealed the lack of DNA repair mechanisms. For the active recovery, a decrease of 54 % of the level of DNA damage has been observed after the first renewal of spermatophores and this level stabilized after the second renewal. On the basis of this second strategy, we defined a mean basal value of 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 highlighted significant abnormal sperm DNA damage within the native population of *P. longirostris* from the Seine estuary on all stations during the six-sampling campaigns.

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

1. Introduction

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

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

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

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

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

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

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

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

2. Materials and methods

2.1. Field collection and maintenance in laboratory conditions

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

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

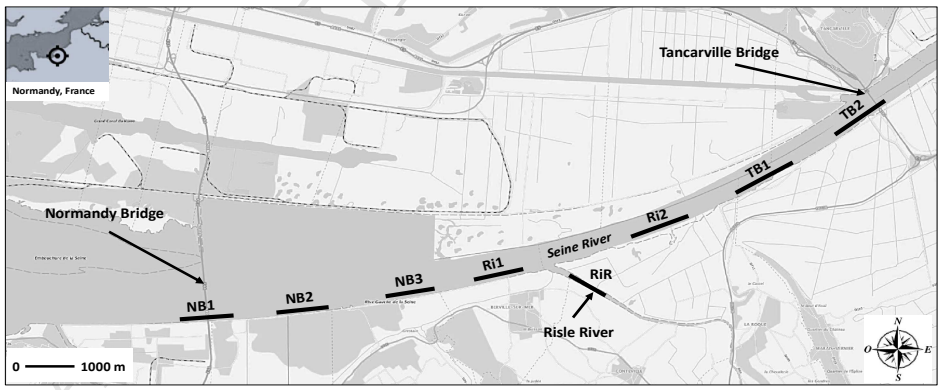


Figure 1. Localization of the sampling stations along the Seine estuary between the Normandy bridge and the Tancarville bridge (Normandie; France). NB1 = Normandie bridge; NB2 = Grestain abbey; NB3 = Grestain; Ri1 = Berville sur mer; RiR = Risle River; Ri2 = Blanc banc; TB1 = Pointe de la Roque and TB2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

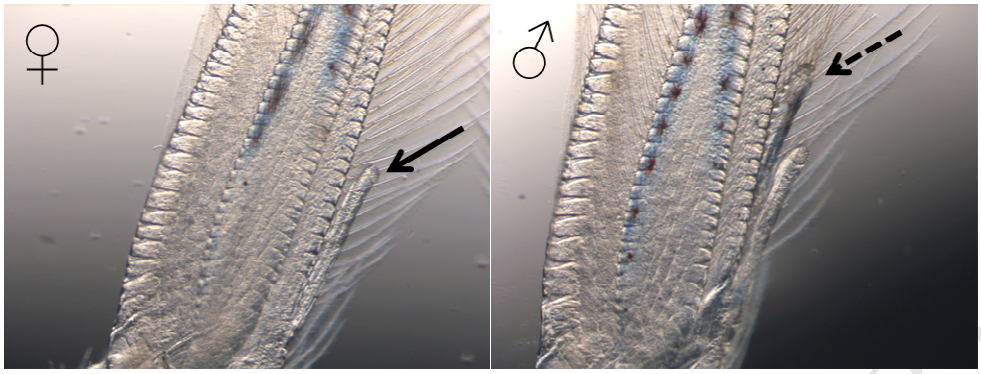


Figure 2. Binocular observation (8x) of the secondary sex characters, the appendix *masculina*, in *Palaemon longirostris* at the second pleopode pair. The continuous arrow marks the appendix *interna* and the hatched arrow marks the appendix *masculina*.

2.2. Procedure of the sperm DNA damage analysis

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

2.3. Experiment of passive recovery in clean artificial sea water

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

(*i.e.* salinity of 10). Mortality was followed every day of this experiment. Every beaker was provided with additional aeration to maintain optimum conditions, and the water was monitored daily for nitrite (NO_2^-) and nitrate (NO_3^-) concentrations which were always included in low values (*i.e.* between 0.01 to 0.02 $mg.L^{-1}$ for nitrite and 5 to 9 $mg.L^{-1}$ of nitrate). The brackish water was renewed every 72 hours. Prawns were fed daily *ad libitum* with pellet B-Penaeus Grower RCE 1 (Le Gouessant®) according to manufacturer's recommendations during the experiment period. Batches of 4 beakers were stopped after 10 and 20 days of recovery (*i.e.* T10 and T20). Sperm samples from each surviving prawn were obtained and individually analysed as described in section 2.4. The DNA integrity was measured only for the sperm samples displaying a cell viability ≥ 85 % (*i.e.* n = 17, 12 and 14 for T0, T10 and T20, respectively).

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

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

2.5. Statistical analysis

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

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

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

3. Results

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

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

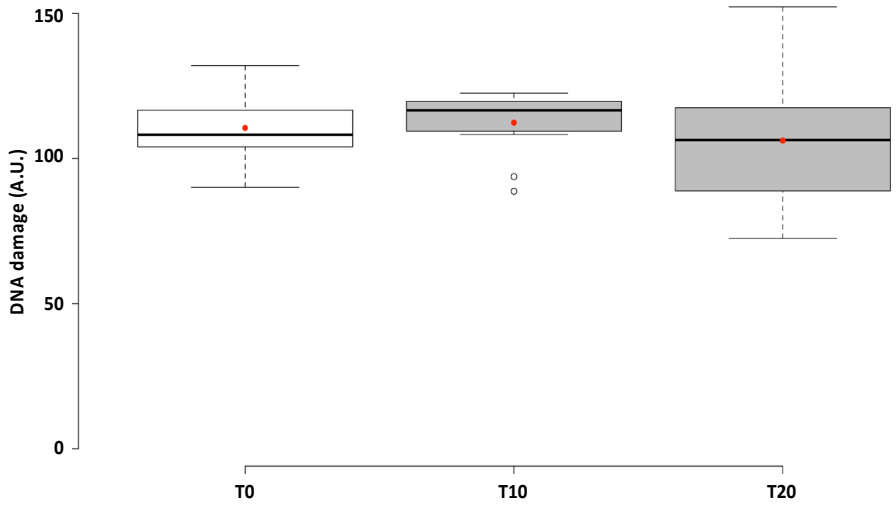


Figure 3. Sperm DNA damage measured using Comet assay in *Palaemon longirostris* specimens sampled in Seine estuary, just before (*i.e.* T0; *n* = 17) and after 10 and 20 days in laboratory recovery period in healthy environment (T10 and T20; *n* = 12 and 14). Results are shown in boxplot (*i.e.* the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

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

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

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

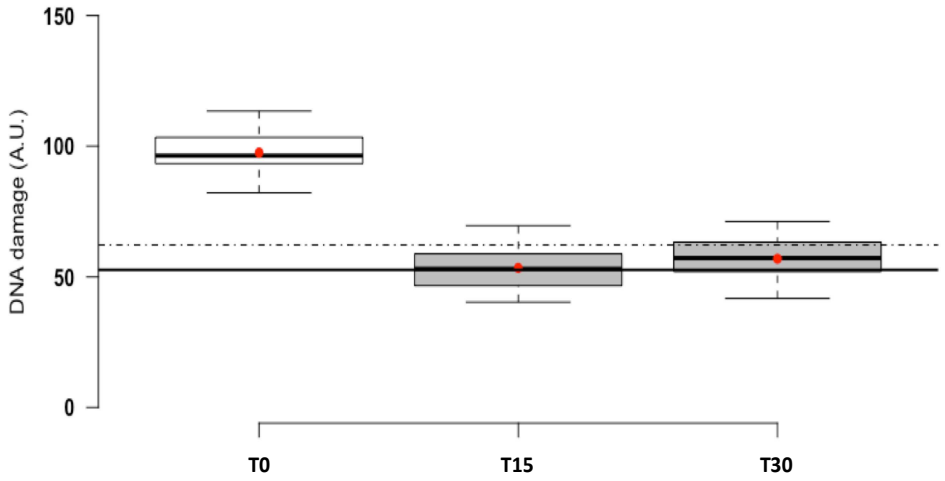


Figure 4. Sperm DNA damage measured using Comet assay in *Palaemon longirostris* specimens sampled in Seine estuary after 3 successive extractions of spermatophores performed just before (*i.e.* T0; n = 17) and after 15 and 30 days of recovery under laboratory conditions in healthy medium (T15 and T30;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

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

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

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

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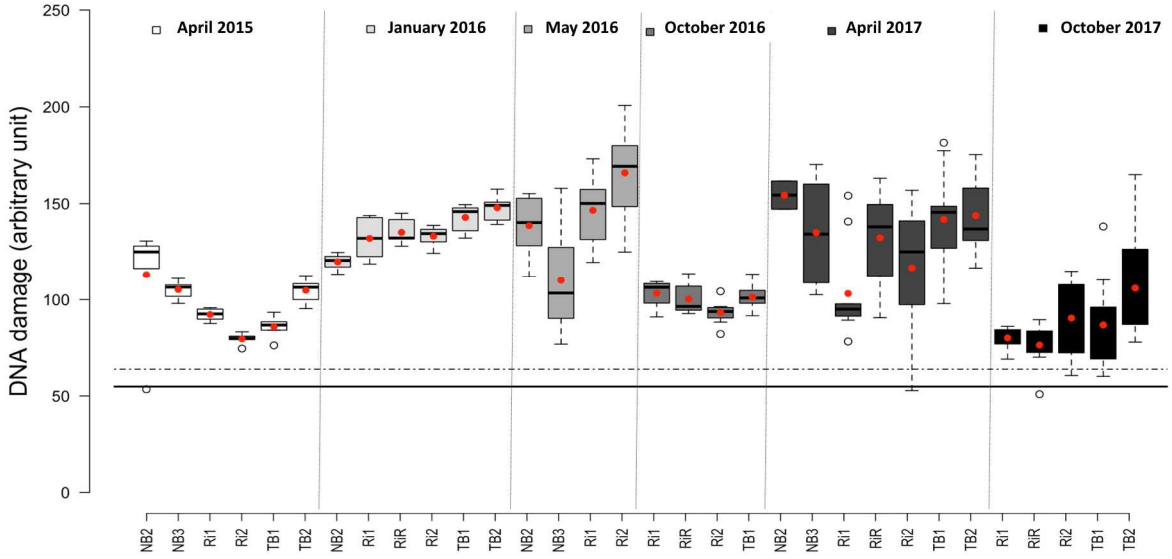

Figure 5. Sperm DNA damage measured using Comet assay in *Palaemon longirostris* specimens sampled in a maximum of 7 stations along the mesohaline part of the Seine estuary during 6 campaigns in 2015, 2016 and 2017. Results are shown in boxplot (*i.e.* the median, the first and the third quartiles, the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

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

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 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 6.4 to 8.1 %, respectively). *In fine*, a significant variability on the level of sperm DNA damage between-month was observed (Scheirer-Ray-Hare pairwise test; p = 2.220446e-16).

4. Discussion

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

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

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

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

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

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

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

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

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

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

Conclusion

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

Acknowledgment

This study was supported by the project ECOTONES funded by the program Seine-Aval V (Public Interest Groups Seine-Aval) (SA5 QO 2014), the Research Federation CNRS 3730 SCALE and the Normandy Region. The authors also thank Mrs. D. Hallidy for proof- reading the English.

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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