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1 **Application of the Fpg-modified comet assay on three-spined**
2 **stickleback in freshwater biomonitoring: toward a multi-**
3 **biomarker approach of genotoxicity**

4

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18

19 **Abstract**

20 Aquatic species are exposed to a wide spectrum of substances, which can compromise their
21 genomic integrity by inducing DNA damage or oxidative stress. Genotoxicity biomarkers as
22 DNA strand breaks and chromosomal damages developed on sentinel species, have already
23 proved to be relevant in aquatic biomonitoring. However, these biomarkers do not reflect DNA
24 oxidative lesions *i.e.* the 8-oxodG, recognized as pre-mutagenic lesion if not or mis-repaired in
25 human biomonitoring. The relevance to include the measure of these lesions by using the Fpg-
26 modified comet assay on erythrocytes of the three-spined stickleback was investigated. An
27 optimisation step of the Fpg-modified comet assay considering both enzyme buffer impact, Fpg
28 concentration and incubation time has been performed. Then, this measure was integrated in a
29 battery of genotoxicity and cytotoxicity biomarkers (considering DNA strand breaks, DNA
30 content variation and cell apoptosis/necrosis and density) and applied in a freshwater
31 monitoring program on six stations of the Artois Picardie watershed (three week-caging of
32 control fish). These biomarkers allowed to discriminate the stations regarding the genotoxic
33 potential of water bodies and specifically by the measure of oxidative DNA lesions, which seem
34 to be a promising tool in environmental genotoxicity risk assessment.

35 **Keywords:** Biomonitoring, Fish, Fpg-modified comet assay, Oxidative DNA damage, DNA
36 strand breaks, DNA content variation, flow cytometer

37

38

39 **Highlights:**

- 40 1- The Fpg-modified comet assay was optimised on stickleback erythrocytes
41
42 2- A multi-biomarker approach of genotoxicity including the Fpg-modified comet assay
43 was applied in a freshwater monitoring
44 3- Oxidative lesions to DNA provided complementary information to DNA strand breaks
45 and chromosomal abnormalities for the freshwater genotoxic risk assessment
46
47

48 1. Introduction

49 In the context of freshwater monitoring programs, the integration of effects-based methods
50 (*i. e.* biomarkers and bioassays) is highlighted under the Water Framework Directive (WFD
51 2000/60/EC) linking ecological and chemical status assessment (Wernersson et al., 2015) to
52 achieve good water quality (Brack et al., 2019b). These key tools located at low levels of
53 biological organisation (*i.e.* sub-individuals, individuals) (Clements, 2000; Lemos, 2021) have
54 the advantage to cover a wide range of exposure and mechanisms of toxicity (Brack et al., 2017,
55 2019a). Among these methods, biomarkers may give an early-warning signals of potential long-
56 term alterations of ecosystems health status (Milinkovitch et al., 2019). Especially, monitoring
57 a set of biological parameters (multi-biomarker approach) allows to assess the global state of
58 the freshwater studied (Galloway, 2006) and already proved its relevance in aquatic
59 biomonitoring programs on various aquatic species (Catteau et al., 2022).

60 Aquatic environments are considered to be the last receptacle of anthropogenic pollutions,
61 making aquatic animals as models of interest to understand the link between pollution and
62 adverse health effects as the increase of cancer at the cellular level (Jha, 2008; Martins and
63 Costa, 2017; Baines et al., 2021). Indeed, aquatic species are exposed to a wide spectrum of
64 substances, which a part can compromise the genomic integrity of cells by inducing DNA
65 damage or by generating oxidative stress (Rhee et al., 2013; da Silva Junior et al., 2021; Ali et
66 al., 2021). These DNA lesions can play a role in mutagenesis and the development of cancer
67 (du Corbier et al., 2005; Lerebours et al., 2017, 2014; Baines et al., 2021). Therefore, genotoxic
68 damages are described as major parameters to be monitored in the assessment of aquatic toxicity
69 (Bolognesi and Cirillo, 2014).

70 Among genotoxicity biomarkers, the detection of primary DNA damage (DNA strand-
71 breaks) and chromosomal-scale lesions (micronucleus) are recommended for instance by the

72 International Council for the Exploration of the Sea (ICES) and are among the main biomarkers
73 used in biomonitoring surveys (Wernersson et al., 2015; Gajski et al., 2019; D'Agostini and La
74 Maestra, 2021). The alkaline comet assay or Single Cell Gel Electrophoresis assay (SCGE) has
75 become one of the methods of choice for the evaluation of primary DNA damage since more
76 30 years (Collins, 2004; Møller, 2018). The alkaline comet assay is a cost effective and sensitive
77 assay to detect both single- (SSBs), double- strand breaks (DSBs) and alkali-labile site (ALS,
78 apurinic/aprimidinic [AP-] sites or baseless sugars) on any individual eukaryotic cell type
79 (Singh et al., 1988; Collins et al., 1997; Collins, 2004; Pu et al., 2015). However, many
80 genotoxic agents do not directly induce strand breaks but can induce other lesions such as
81 oxidized bases, which are not detected by the comet assay in its alkaline version (Muruzabal et
82 al., 2021). To overcome this, the comet assay can be coupled with an enzyme of the base
83 excision repair (BER) system to assess oxidative and alkylating DNA damage (Collins, 2014;
84 Muruzabal et al., 2021). Among the three most selected enzymes (Endonuclease III (Endo III),
85 formamidopyrimidine DNA glycosylase (Fpg), 8-oxoguanine DNA-glycosylase (hOGG1)); the
86 Fpg, a N-glycosylase and AP-lyase from *Escherichia coli*, is the most used in ecotoxicological
87 studies (Muruzabal et al., 2021). Indeed, Fpg recognizes a wide spectrum of DNA lesions (net
88 Fpg-sensitive sites) including oxidized purines as 8-oxo-7,8-dihydroguanine (8-oxoGua)
89 among the most abundant (Angeli et al., 2011; Azqueta et al., 2009; Boiteux, 1993) and ring-
90 opened N7 guanine adducts (N-7 alkylguanines) alkylating damages in DNA (Speit et al., 2004;
91 Azqueta and Collins, 2013). The Fpg removes then the AP-site created leaving 1-base gap
92 (Boiteux, 1990; Muruzabal et al., 2021) making additional strand breaks detectable by the
93 alkaline comet assay.

94 The sensitivity of the Fpg-modified comet assay explains its wide use in toxicology and
95 human biomonitoring, but remains less frequently applied in environmental and ecotoxicology
96 studies (Muruzabal et al., 2021). In aquatic ecotoxicology, some studies have already

97 investigated the application of the Fpg-modified comet assay on fish cell lines (Žegura and
98 Filipič, 2019). Notably, the Fpg-comet assay has been performed with the *in vitro* exposure of
99 fish cell lines to model genotoxicant (methylmethanesulfonate, hydrogen peroxide), or to
100 environmental contaminants (B[a]P, cadmium, herbicides diuron) (Kienzler et al., 2012) and
101 showed a higher sensitivity in comparison to the alkaline version (Kienzler et al., 2012; Amaeze
102 et al., 2015). In aquatic biomonitoring, the Fpg-modified comet assay has been adapted on
103 different models, such as fish erythrocytes (*i.e. Alburnus alburnus, Squalius cephalus, Alburnus*
104 *alburnus*) (Kolarević et al., 2016, 2018; Jovanović et al., 2018) or hemolymph of *Daphnia*
105 *magna* (Pellegrini et al., 2020). These studies have highlighted the toxic effect of substances
106 causing oxidative damage to DNA and offered a more complete picture of the genotoxic
107 potential of xenobiotics (Araldi et al., 2015).

108 Fish considered as good bioindicators of aquatic pollution, is the most important group of
109 sentinel species for aquatic monitoring (Gajski et al., 2019). In these species, the sampling of
110 erythrocytes is easy (Osman, 2014), giving access to a high density of already isolated and
111 nucleated-cells (Witeska, 2013). Peripheral erythrocytes responsible for oxygen transport are
112 the main actors for redox reactions that produce reactive oxygen species (ROS), which can
113 affect the DNA integrity and lead to eryptosis (Frag and Alagawany, 2018; Lemos, 2021).
114 These advantages make erythrocytes particularly attractive and suitable cell model to assess
115 specifically the genotoxicity induced by xenobiotics (Shiva Shankar Reddy et al., 2007; Frag
116 and Alagawany, 2018). Among small-bodied teleost fish, the three-spined stickleback
117 *Gasterosteus aculeatus*, an euryhaline species of the northern hemisphere (Wootton, 1976) is
118 increasingly used as sentinel species in active biomonitoring studies (Le Guernic et al., 2016b;
119 Catteau et al., 2020, 2021, 2022). A wide array of biomarkers has been developed and validated
120 in stickleback, including innate immune responses, endocrine disruption, neurotoxicity,
121 oxidative stress and genotoxicity biomarkers (Sanchez et al., 2008; Katsiadaki et al., 2012;

122 Bado-Nilles et al., 2013; Santos et al., 2014; Marchand et al., 2017). Genotoxicity biomarkers
123 as DNA strand breaks or DNA content variation considered alone, have already proven to be
124 relevant in environmental biomonitoring survey (Santos et al., 2016; Le Guernic et al., 2016a;
125 Slaby et al., 2023). Together, these two genotoxicity biomarkers included to the battery of
126 biomarkers performed in stickleback allowed a better discrimination of degraded stations (Cant
127 et al., 2022). However, oxidative and alkylating DNA damage were never monitored in
128 stickleback. The relevance to include a biomarker covering the lesions revealed by the Fpg-
129 modified comet assay, like the 8-oxodG as the main DNA oxidative lesion and recognized as a
130 pre-mutagenic lesion in human biomonitoring, was investigated in this study. Our goal is to
131 propose at term a more comprehensive approach of environmental genotoxicity by considering
132 in sentinel species, various types of DNA damage which may occur at different genomic scale.

133 After set-up Fpg-modified comet assay protocol in erythrocytes of the three-spined
134 stickleback, its applicability through an active biomonitoring context was investigated on the
135 Artois-Picardie watershed (north France), following the caging conditions as already performed
136 in previous studies (Le Guernic et al., 2016b; Catteau et al., 2019). The DNA lesions detected
137 by the Fpg-modified comet assay were compared with the response of other genotoxicity
138 biomarkers (DNA strand breaks and DNA content variation) already developed in stickleback
139 as well as erythrocyte mortality and density (Le Guernic et al., 2016a; Santos et al., 2016; Cant
140 et al., 2022).

141

142 **2. Materials and Method**

143 **2.1. Fish blood sampling**

144 Each experiment was conducted in accordance with European directive 2010/63/UE on the
145 protection of animals used for scientific purposes at INERIS facilities (registration number E60-
146 769-02, APAFIS #20760). For the present work, all sticklebacks came from a well-
147 characterized population of the French National Institute for Industrial Environment and Risks
148 (INERIS) (Verneuil-en-Halatte, Oise, France) which were maintained in outdoor breeding
149 facilities with natural vegetation and macro-invertebrate communities.

150 Each stickleback was directly anesthetised by balneation with MS222 (Tricaine
151 methanesulfonate, 100 mg/L, PHARMAQ Fishteq AS, Norway) to avoid handling stress and
152 killed by cervical dislocation before to be weighed, measured (standard length) and sexed. Then
153 for each fish, 2 μ L of blood sample were immediately collected from the cervical cut, and
154 directly diluted in 120 μ L of citrate buffer (85.5 g/L of sucrose (Sigma), 11.76 g/L of trisodium
155 citrate dihydrate (Sigma), 50 mL/L of DMSO (Sigma), pH: 7.6)) (Vindeløv and Christensen,
156 1990). The same blood sample was used to measure all biomarkers of genotoxicity and
157 erythrocyte markers. Precisely, 50 μ L of the diluted blood sample were maintained at 4 ± 1 °C
158 until erythrocyte density and mortality analysis (the day after sampling for the field study). The
159 rest of blood (50 μ L for cytometry and 20 μ L for comet assays) was directly cryopreserved in
160 liquid nitrogen. These last samples were stored at - 80°C until DNA content variation, DNA-
161 strand breaks and oxidative DNA damage analysis.

162

163 **2.2. Optimisation of the FPG-modified comet assay on peripheral erythrocytes**

164 DNA damage was assessed by the Fpg-modified comet assay on cryopreserved erythrocytes
165 of stickleback. The protocol has been adapted according to previous assays performed on

166 human lymphocytes (Collins et al., 1996) as well as on fish erythrocytes (Pannetier et al., 2019).
167 This optimisation consisted to test the suitability of the enzyme buffer [0.1 M KCl, 40 mM
168 HEPES, 0.5 mM EDTA, 0.2 mg/mL BSA, 20mg/mL, pH 8], to define the concentration (0, 0.5
169 and 1 U/mL tested) and the incubation time (30 and 45 min tested) of the Fpg. Then, the
170 sensibility of our erythrocyte model was tested by using a reference genotoxicant, the Ro19-
171 8022 plus light. For optimisation experiments, ten specimens were used to study the incidence
172 of enzyme buffer, six specimens for the incidence of Fpg (time and concentration) on basal
173 DNA strand breaks, and four specimens for the positive control. Currently, the analyses were
174 realized at 4°C under inactinic light (dim red light) to avoid supplementary DNA damage. The
175 blood samples were rapidly defrosted (18s) in a 37°C-bath, fiftieth (1/50) diluted in PBS buffer
176 (0.01 M, pH 7.4, without neither Ca nor Mg) to adjust cell density, and finally half-diluted with
177 agarose LMP (Low Melting Point, – type VII, 1% w/v in PBS buffer, 0.01 M, pH 7.4, without
178 Ca or Mg) maintained in liquid state at 37°C. Three conditions for each sample were performed
179 with 2 gels of 30 µL / slide for the simultaneous measure of both DNA strand breaks (by alkaline
180 comet assay), enzyme buffer (Fpg-comet assay without the enzyme) and DNA strand breaks
181 plus net Fpg-sensitive sites (Fpg-comet assay with the enzyme). After chilling (15 min on ice)
182 and coverslips removed, embedded erythrocytes were treated in a cold lysing solution, pH 10
183 for one hour. Then, nucleoids on gels designed for the measure of Fpg-specific lesions (enzyme
184 buffer and Fpg slides) were twice washed (30 second each) at 4°C in demineralized water and
185 once (15 min) at 4°C with the enzyme buffer (0.1 M KCl, 40 mM HEPES, 0.5 mM EDTA,
186 0.2 mg/mL BSA (New England, BioLabs®, 20mg/mL)) at pH 8. On each gel, 80 µL was
187 deposited corresponding to either the buffer (without Fpg) or Fpg enzyme tested at
188 concentrations of 0, 0.5 and 1 U/mL (New England Biolabs®, stock solution at 8 000 U/mL).
189 These slides were covered with a squared cover slip of parafilm to spread the buffer (with or
190 without Fpg) homogeneously over the agarose layer and placed in a moist box at 37°C. The

191 innocuity of the enzyme buffer was checked at 30 or 45 min of incubation based on literature
192 data (Zhao et al., 2015; Jovanović et al., 2018; Pannetier et al., 2019). After incubation, gels
193 have been resolidified at 4°C (15 min) and then cover slip of parafilm removed. The
194 photosensitizer Ro19-8022 plus light has been selected as a positive assay control of the Fpg-
195 modified comet (Collins, 2014; Møller et al., 2018). Based on protocols in human cells (Møller
196 et al., 2018), fish blood cells were *ex vivo* exposed to 200 µL of the Ro19-8022 at 1 µM (Chiron,
197 stock solution 100µM) for 2.5 or 5 min of irradiation on ice (at a distance of 25 cm under
198 1000 W-light).

199 All slides – DNA strand breaks, enzyme buffer and DNA strand breaks plus Fpg-sensitive
200 sites – were washed together twice (30 seconds each) at 4°C in demineralized water before both
201 the denaturing step in an alkaline buffer (pH > 13) for 40 min and the electrophoresis
202 (0.8 V.cm⁻¹, 24 min, 240 mA, 5 W). Then, slides were washed in a neutralization buffer (twice
203 20 min), dried with the absolute ethanol (10 min) and kept at room temperature in the dark until
204 their staining with Sybr Green 1X and scanning (EVOSTM FL Auto 2, InvitrogenTM Thermo
205 Fisher Scientific, Bothell WA, USA, optical filter for Sybr Green (497 nm / 520 nm)). For each
206 slide, a total of 200 comets by conditions (corresponding to 100 nucleoids / gel with two gels
207 for one slide for each condition) was scored with the Comet Assay IV software (version 4.3.1,
208 Instem, Staffordshire, UK). To express DNA damage, the percentage of tail intensity (TI %) was
209 chosen according to Langie et al. (2015). For each gel (100 comets) the median of tail intensity
210 was calculated. Comet assay results by conditions were expressed as the mean of the two gel
211 medians (200 comets). The net level of Fpg-sensitive sites was obtained by subtracting the
212 values of damages obtained from the alkaline comet assay and the buffer enzyme, as [Net Fpg-
213 sensitive sites = TI % Fpg-modified comet assay - TI % Alkaline comet assay].

214

215 **2.3. Using the Fpg-modified comet assay for freshwater biomonitoring**

216 In September/October 2020, 156 one-year-old sticklebacks with homogeneous length
217 (44.83 ± 0.57 mm) and weight (1.27 ± 0.08 g) were sexed (De Kermoisan et al., 2013) and
218 caged for 21 days by batch of 26 fish in a 36 L cylindrical stainless-steel tanks in six distinct
219 stations in the north of France (Fig. 1). These caging conditions (fish density and no external
220 food supply) were recommended as inducing no effect on biomarkers responses (Le Guernic et
221 al., 2016b; Catteau et al., 2019). The Cligneux river at Saint-Rémy du Nord (“*St Rémy du Nord*
222 ” station), the Rhonelle river at Artres (“*Artres*” station,) the Scarpe river at Biache-Saint-Vaast
223 (“*Biache-Saint-Vaast*” station), the Deule river at Courrières (“*Courrières*” station and the
224 Sensée river at Bouchain (“*Bouchain*” station) were previously described in Cant et al. (2022).
225 All these sites are subjected to various anthropogenic pressures and contaminated by polycyclic
226 aromatic hydrocarbons (PAHs), considered as downgrading substances (WFD, 2000/60/CE)
227 (Agence de l’Eau Artois-Picardie 2020). A watercress crop at Manqueville (“*Manqueville*”
228 station) not yet studied was added to the experiment as a reference station located in the same
229 watershed. Twenty-six fish from the initial population of the outdoor pond at Verneuil-en-
230 Halatte (“*Verneuil*” station) were also added as control condition. In addition to this study, the
231 effect of caging on each erythrocyte biomarkers was investigated as previously done for the
232 other biomarkers measured in sticklebacks (Catteau et al., 2019). In this sense, a different batch
233 of fish (October 2021) was divided into two groups of 30 specimens (30 free and 29 caged fish
234 collected after 21 days) from the same outside breeding pond.

235
236 In each station, temperature was monitored continuously using a probe hooked to the
237 cage (HOBO Pendant Temperature Data Logger, Onset, Bourne, MA, USA). Water, pH and
238 conductivity were measured at the first day and last day of exposure in each river by using a
239 WTW multi-parameter instrument equipped with digital sensors (MultiLine® IDS, Multi3430,
240 WTW, Germany). The temperatures measured were similar for all stations ranging from 11.25

241 ± 0.90 °C for *St Rémy du Nord* to 14.84 ± 1.09 °C for control pool. The pH of water oscillated
242 between 7.77 ± 0.23 and 8.34 ± 0.34 . The lowest conductivity was measured in the reference
243 station (395 ± 40 $\mu\text{S}/\text{cm}$) and the highest in *Courrières* (755 ± 40 $\mu\text{S}/\text{cm}$). These physico-
244 chemical parameters were detailed in Table 1.

245 After caging, sticklebacks were dissected directly *in situ* to avoid transport stress (Le
246 Guernic et al., 2016b). After blood sampling, the detection of DNA strand breaks and net Fpg-
247 sensitive sites was carried out (see Protocol section 2.2 and Results section 3.1). In complement,
248 the general well-being of sticklebacks was evaluated by the Fulton's condition index. The
249 measure of peripheral erythrocyte density, mortality and chromosomal aberration was
250 performed following the protocols described in Cant et al. (2022). Briefly, all analyses were
251 performed after retired duplicate erythrocytes (SSC-H / SCC-A) by flow cytometry. The
252 erythrocyte counts were detected using forward scatter (FSC—size of cells) and side scatter
253 (SSC—complexity of cells) parameters. Erythrocyte mortality was detected using a double
254 labelling method with YO-PRO®-1 (1 mM in DMSO, Invitrogen) for apoptotic cells (FITC-H,
255 green fluorescence) and propidium iodide (1 mg/mL in water, Invitrogen) for necrotic cells on
256 fresh erythrocytes (PERCP-Vio700-H, red fluorescence) as preconized for leucocytes by Bado-
257 Nilles et al. (2014). After 10 min of incubation on ice and in darkness, samples were analyzed
258 after cell excitation by a 488 nm argon laser by FCM (25 000 events) (MacsqantX, Miltenyi
259 Biotec, USA).

260 Chromosomal damage was determined on erythrocytes according to the original method
261 developed by Vindeløv and Christensen (1990) and adapted by Marchand et al. (2017) on
262 stickleback. Erythrocytes densities were adjusted at $40 \cdot 10^6$ cells/mL. Successive treatments
263 were applied to disrupt cytoplasmic and nuclear membranes to disrupt RNA and access DNA,
264 stabilize amino acids and mark DNA by the propidium iodide (1 mg/mL in water, Invitrogen,
265 Carlsbad, CA, United States). A sample of stabilized chicken red blood cells (Fitzgerald

266 Industries, Acton, United States) was used as a standard and analyzed simultaneously. Each
267 PerCP-Vio700-A peak coefficient of variation (CV) corresponds to DNA content variation,
268 referred to chromosomal aberrations. DNA damage corresponds to the CV of fish erythrocyte
269 samples minus the CV of chicken red blood cell samples (Easton et al., 1997). All samples were
270 analyzed by FCM (30 000 events) (MacsqantX, Miltenyi Biotec, USA).

271

272 **2.4. Statistical analysis**

273 The R software version 3.6.1 was used to perform all statistical analyses of this study. Two
274 kinds of tests were carried out. To evaluate a significative difference on genotoxic biomarkers
275 between two groups only, for the part optimisation (adapted for paired or independent data) and
276 for the comparison of each biometric index (K, weight, standard length) between T0 and T21
277 (independent samples) in the field application, a Student t-test was applied for each biomarker
278 after checking the normality (Shapiro-Wilk's test) and homoscedasticity (Leneve's test) of raw
279 data or after a log-transformation (if the normality and homoscedasticity were not met with raw
280 data). If these criteria were not met despite the log-transformation, a Wilcoxon-Mann-Whitney
281 test was performed.

282 To assess the difference between the biomarker's levels of more than two groups, a one-
283 way ANOVA type III was carried out to compare each Fpg concentration and each cell exposure
284 condition for the Ro19-8022 validation (paired data, Part Optimisation). Normality (Shapiro-
285 Wilk's test) of raw data were checked to validate the use of an ANOVA (*anova_test ()* function
286 of package *rstatix*). A Bonferroni's test was performed as post hoc. A two-way ANOVA type
287 III for unbalanced plan (independent data, Part Field application) with "Station" and "Sex" as
288 factors was carried out to analyse genotoxicity biomarkers responses between each station.
289 Normality (Shapiro-Wilk's test) and homoscedasticity (Leneve's test) of ANOVA residuals

290 were checked to validate the use of an ANOVA (*lm ()* function of R package *car*) (on raw or
291 log-transformed data). A Tukey's test was performed as post hoc analyses (*TukeyHSD ()*
292 function of R package *stats*). If the conditions to perform ANOVA analyses were not met (no
293 normality neither homoscedasticity), a Kruskal-Wallis test was applied followed by adapted
294 post hoc tests for non-parametric data (packages *agricolae*).

295 Finally, a standardized principal component analysis (PCA) was performed to illustrate the
296 discrimination of stations with all biomarker responses on the two first principal dimensions
297 (package *FactoMine R*). All hypotheses were tested at the level of $p \leq 0.05$. The statistical
298 analyses are detailed in the Tables Supplementary data 1 to 8.

299 3. Results

300 3.1. Optimisation of the FPG-modified comet assay on blood erythrocytes

301 The first optimisation step was to check the suitability of the enzyme buffer on levels of
302 DNA damage in erythrocytes. According to preliminary studies, the enzyme buffer obtained
303 with the protocol of Collins et al., (1996) [0.1 M KCl, 40 mM HEPES, 0.5 mM EDTA,
304 0.2 mg/mL BSA, pH 8] was selected, despite a significant increase of DNA strand breaks on
305 erythrocytes of our fish species (Table 2, Supp. data 1). In mean, the incidence of enzyme buffer
306 was estimated at 6.56 ± 5.78 TI % based on the analysis of erythrocytes from 20 fish (Table 4).
307 The optimal Fpg concentration was studied in a second step, highlighting that the measurement
308 of DNA lesions, after incubation with Fpg for 30 min at 37°C, was significantly increased at
309 1 U/mL (18.70 ± 6.60 TI %) compared to the 0.5 U/mL (16.73 ± 5.22 TI %) and to the basal
310 level under the alkaline comet assay conditions (10.06 ± 2.29 TI %) (p -value = 0.003, Table 2,
311 Supp. data 2). Thus, the protocol parameters were selected by choosing an incubation of
312 erythrocytes with Fpg at 37°C for 30 min at the concentration of 1 U/mL. A higher Fpg
313 concentration (tested up to 2.5 U/mL) or time of treatment (45 min) did not improve the
314 detection of DNA lesions, as demonstrated by a preliminary experiment (Data not shown).

315 The last step was to validate this protocol by the exposure of erythrocytes to the
316 photosensitizer Ro19-8022 plus irradiation, as recommended as controls for monitoring Fpg-
317 sensitive sites (Collins, 2014; Møller et al., 2018). Addition of the Ro19-8022 without
318 irradiation (with Fpg) did not significantly increase the level of DNA strand breaks
319 (17.44 ± 2.43 TI %, Table 3), which is at the same range as the ones obtained in alkaline
320 condition (without Fpg) (12.13 ± 3.19 TI %, Table 3) and with the Fpg (20.22 ± 4.06 % TI,
321 Table 3). The activation of the photosensitizer by light for 2.5 min significantly increased the
322 rate of DNA damage at 47.78 ± 2.05 TI % to reach 62.52 ± 10.68 TI % after 5 min of
323 erythrocyte's irradiation (p -value = $2.99e^{-08}$, Supp. data 3). The rate of DNA damage was

324 multiplied by 2.7 and 3.6 after 2.5 and 5 min of irradiation of erythrocytes with the Ro19-8022
325 plus light, respectively. No significant increase of highly DNA damaged-cells (hedgehogs) was
326 observed with these conditions (Table 3). The exposure to the Ro19-8022 plus irradiation
327 confirmed the sensitivity of erythrocyte DNA to oxidative lesions detected with our protocol
328 (1 U Fpg/mL for 30 min at 37°C). These parameters were applied on all the experiments
329 presented below.

330

331 **3.2. Evaluation of the caging impact on genotoxic biomarkers developed**

332 To ensure the applicability for active biomonitoring, the caging impact was evaluated for
333 the entire genotoxic biomarkers developed on peripheral blood cells of sticklebacks.

334 Caging of fish did not induce significant effect on erythrocyte apoptosis (7.31 ± 10.29 %, p -value = 0.89), Fpg-sensitive sites (6.54 ± 5.77 TI %, p -value = 0.43), DNA strand breaks
335 (9.38 ± 4.58 TI %, p -value = 0.47) and DNA content variation (1.78 ± 1.52 CV, p -value = 0.41)
336 compared to “free fish” (6.35 ± 7.53 %; 7.67 ± 4.71 TI %, 8.37 ± 3.91 TI %; 1.69 ± 1.13 CV;
337 1.69 ± 1.13 CV, respectively; Table 4, Supp. data 4), despite a lower standard length and weight
338 of caged fish. Only the density ($2.18 \times 10^5 \pm 1.55$ cell / mm³, p -value = $1.13e^{-08}$) and necrosis
339 (5.68 ± 5.07 %, p -value = $2.97e^{-06}$) of erythrocytes were significantly lower in caged fish
340 ($4.28 \times 10^5 \pm 1.01$ cell / mm³, 13.87 ± 8.00 %, respectively; Table 4, Supp. data 4).

342

343 **3.3. Freshwater biomonitoring by application of the Fpg-modified comet assay**

344 The number of sticklebacks collected after 21 days of exposure and all responses of
345 biomarkers for each group of fish in each station are shown in Table 5 (mean \pm sd). These raw
346 values were used for all the analyses presented below. Out of 123 caged sticklebacks were

347 collected from the field, with 27.64 % of males and 72.36 % of females. No significant impact
348 of gender was demonstrated on any of the parameters studied (p -values > 0.05, Supp. data 5).
349 The survival rate of fish was 96.15 % in *Artres*, 92.31 % in *Bouchain*, 88.46 % in *Manqueville*
350 as in *St Rémy du Nord*, 69.23 % in *Biache-Saint-Vaast* and 38.46 % in *Courrières*, where a high
351 individual mortality was recorded (Table 5).

352 The study of the PCA provided information on the distribution of fish based on the
353 contribution of each biomarker. In the present work, 48.6 % of the overall variance in the two
354 principal components of the PCA (Fig. 2, Supp. data 6) was explained in the first axis (26.5 %)
355 especially by erythrocyte apoptosis (44.66 % of contribution) and necrosis (27.89 % of
356 contribution), DNA content variation (11.90 % of contribution) and erythrocyte density
357 (11.30 % of contribution) and in the second axis (22.1 %) mainly due to net Fpg-sensitive sites
358 (31.26 % of contribution), DNA strand breaks (24.66 % of contribution), erythrocyte necrosis
359 (17.95 % of contribution) and fresh erythrocyte (14.93 % of contribution) (Fig. 2, Supp. data 6).
360 The first group of the PCA concern reference stations (*Verneuil*, *Manqueville*) (Fig. 2, Table
361 5). This reference group was characterised by the highest erythrocyte densities ($6.03 \pm 3.90 \times$
362 10^5 cell/mm³ at *Verneuil* and $4.71 \pm 3.13 \times 10^5$ cell/mm³ at *Manqueville*) associated with an
363 erythrocyte mortality characterised by a strong inter-individual variability (*Verneuil*:
364 11.53 ± 9.58 % for necrosis and 7.16 ± 4.16 % for apoptosis; *Manqueville*: 14.91 ± 11.62 % for
365 necrosis and 4.91 ± 6.68 % for apoptosis). Overall, the genotoxic parameters were lower than
366 in other groups (DNA strand breaks: 8.18 ± 2.96 TI % at *Verneuil*, 6.80 ± 2.38 TI % at
367 *Manqueville*; Fpg-sensitive sites: 7.64 ± 6.79 TI % at *Verneuil*, 5.53 ± 3.66 TI % at
368 *Manqueville*; DNA content variation: 0.30 ± 0.59 CV at *Verneuil*, 0.51 ± 0.49 CV at
369 *Manqueville*). *Manqueville* was also characterised by a significant increase of Fulton's
370 condition index observed after the exposure (from 1.41 ± 0.13 to 1.50 ± 0.15 , p -values = 0.042,
371 Table 6, Supp. data 7) and a significant decrease of erythrocyte apoptosis in caged fish at

372 *Manqueville* (4.91 ± 6.88 %) compared to free fish from *Verneuil* (7.16 ± 4.16 %) was
373 highlighted (p -value = $1.491e^{-12}$, Table 5, Supp. data 5).

374 The two other groups concern fish from the downgraded stations. Except at *Courrières*
375 corresponding to the station characterised by the highest fish mortality, a significant decrease
376 of the Fulton's condition index (K) was shown after 21 days of caging (*St Rémy du Nord* : from
377 1.50 ± 0.18 to 1.24 ± 0.17 , p -values = $5.44e^{-06}$; *Artres* : from 1.40 ± 0.16 to 1.27 ± 0.13 , p -
378 values = $4,69e^{-3}$; *Biache-Saint-Vaast* : from 1.42 ± 0.18 to 1.32 ± 0.13 , p -values = $1.92e^{-2}$;
379 *Bouchain* : from 1.32 ± 0.11 to 1.24 ± 0.09 , p -values = $6.53e^{-3}$), which could be due to weight-
380 loss as significantly observed at *St Rémy du Nord* (16.91 % of decreasing, p -values = $2.64e^{-3}$)
381 and *Artres* (10.61 % of decreasing, p -values = $2.35e^{-2}$). More specifically, a second group
382 gathered the fish caged at *Biache-Saint-Vaast* and *Courrières* (Fig. 2, Table 5). These
383 sticklebacks were mainly characterised by high data of erythrocyte mortality (*Biache-Saint-*
384 *Vaast*: 20.64 ± 8.13 % for necrosis and 4.51 ± 4.51 % for apoptosis; *Courrières*: 20.10 ± 8.85 %
385 for necrosis and 9.45 ± 4.87 % for apoptosis), by a trend towards to increase for DNA strand
386 breaks (12.48 ± 5.73 TI % at *Biache-Saint Vaast*; 11.80 ± 6.08 TI % at *Courrières*) and by high
387 net Fpg-sensitive sites (13.60 ± 5.03 TI % at *Biache-Saint-Vaast* and 23.43 ± 10.93 TI % at
388 *Courrières*). This group was also characterised by low erythrocyte density ($3.63 \pm 1.36 \times 10^5$
389 cell/mm^3 at *Biache-Saint-Vaast* and $2.17 \pm 0.96 \times 10^5 \text{ cell}/\text{mm}^3$ at *Courrières*).

390 A last group formed by the fish caged at *St Rémy du Nord* , *Artres* and *Bouchain* was also
391 characterised by lower values whatever the biomarker considered (erythrocyte density :
392 $2.72 \pm 0.53 \times 10^5 \text{ cell}/\text{mm}^3$ at *St Rémy du Nord* , $3.25 \pm 0.72 \times 10^5 \text{ cell}/\text{mm}^3$ at *Artres* and
393 $3.63 \pm 1.36 \times 10^5 \text{ cell}/\text{mm}^3$ at *Bouchain* ; necrosis : 1.01 ± 0.59 % at *St Rémy du Nord* ,
394 6.20 ± 8.39 % at *Artres* , 5.03 ± 5.22 % at *Bouchain* ; apoptosis : 0.45 ± 0.67 % at *St Rémy du*
395 *Nord* , 2.17 ± 5.11 % at *Artres*, 2.23 ± 3.12 % at *Bouchain* ; DNA strand breaks :
396 9.81 ± 3.58 TI % at *St Rémy du Nord* , 11.87 ± 4.52 TI % at *Artres*, 10.06 ± 4.15 TI % at

397 *Bouchain*; net Fpg-sensitive sites : 12.21 ± 5.56 TI % at *St Rémy du Nord*, 9.07 ± 5.98 TI % at
398 *Artres* and 9.47 ± 7.59 TI % at *Bouchain*).

399 A significant increase in DNA strand breaks levels have been demonstrated only for fish
400 caged at *Artres* and *Biache-Saint-Vaast* compared to the basal level obtained in the control pond
401 *Verneuil* and in the reference station *Manqueville* (11.87 ± 4.52 TI %, 12.48 ± 5.73 TI %, 8.18 ± 2.96 TI % and 6.80 ± 2.38 TI % respectively, p -values = $1.13e^{-2}$, Table 5, Supp. data 5),
402 whereas additional DNA damage have been highlighted with the inclusion of Fpg. The latter
403 were characterized by a significant increase of DNA strand breaks (with Fpg) for sticklebacks
404 from *St Rémy du Nord*, *Artres*, *Biache-Saint-Vaast* and *Courrières* compared to the basal levels
405 assessed in fish from *Verneuil* and *Manqueville* (22.02 ± 6.62 TI %, 20.93 ± 6.95 TI %, 25.72 ± 6.37 TI %, 35.23 ± 15.51 TI %, 15.72 ± 5.93 TI %, 12.27 ± 3.72 TI %; respectively,
406 p -values = $1.12e^{-10}$, Table 5, Supp. data 5). The discrimination of station was improved by the
407 integration of DNA strand breaks measured with Fpg compared to the levels obtained without
408 Fpg (Table 5). Indeed, fish caged in *Courrières* were characterized by a significant rise of net
409 Fpg-sensitive sites (23.43 ± 10.93 TI %) compared to the two reference stations (7.64 ± 6.79 TI
410 % at *Verneuil* and 5.53 ± 3.66 TI % at *Manqueville*, p -values = $4.45e^{-07}$, Table 5, Supp. data 5).
411 A significant induction of net Fpg-sensitive sites was also found in fish from *St Rémy du Nord*
412 and *Biache-Saint-Vaast* but only compared to *Manqueville* (12.21 ± 5.56 % TI, 13.60 ± 5.03 %
413 TI, 5.53 ± 3.66 TI %, respectively, p -values = $4.45e^{-07}$, Table 5, Supp. data 5).

416 The DNA content variation was the biomarker contributing the least to the construction of
417 this PCA representation (11.90 % of contribution, Fig. 2, Supp. Data 6), although it allowed to
418 highlight two stations characterised by chromosomal damages. Fish caged at *Artres* ($1.14 \pm$
419 0.62 CV) and *Biache-Saint-Vaast* (1.06 ± 0.55 CV) exhibit a significant increase of DNA
420 content compared to *Courrières*, (0.19 ± 0.60 CV), *Bouchain* (0.44 ± 0.66 CV) and the
421 reference stations (0.30 ± 0.59 CV for *Verneuil* and 0.51 ± 0.49 CV for *Manqueville*, p -values

422 = 6.68e-07, Table 5, Supp. Data 5). A part of fish from *St Rémy du Nord* (0.86 ± 0.88 CV) was
423 impacted by a non-significant increase of chromosomal abnormalities.

424

425

426 4. Discussion

427 The measurement of DNA damage on aquatic organisms is appropriated to assess genotoxic
428 contamination of freshwater environments. Currently, the evaluation of the primary DNA
429 strand breaks is proposed by the alkaline comet assay (Collins, 2004; Møller, 2018). Since many
430 genotoxic agents can induce other lesions such as oxidized bases, the development of the
431 Fpg-modified comet assay was proposed on sticklebacks in the present work. After an
432 optimisation phase (time exposure, concentration of the Fpg enzyme and the incidence of
433 caging), the protocol was tested with other genotoxic biomarkers previously developed (DNA
434 strand breaks and DNA content variation) on six stations of the north of France by using active
435 biomonitoring.

436 The optimisation of the Fpg-modified comet assay allowed to choose an incubation of
437 erythrocytes with a Fpg concentration of 1 U/mL for 30 min at 37°C, with the enzyme buffer
438 proposed by Collins et al. (1996) [0.1 M KCl, 0.5 mM EDTA, 40 mM HEPES, 0.2 mg/mL
439 BSA, pH = 8]. All the recommendations concerning the optimisation of the enzymatic
440 concentration and the duration of incubation of the Fpg enzyme defined by Møller et al. (2020)
441 were therefore respected to validate the protocol on our cell model. However, the protocol of
442 the Fpg-modified comet assay is not harmonized on fish erythrocytes and appears to be species
443 dependent. Indeed, different concentrations of the Fpg, time incubation and enzyme buffer can
444 be used according to the literature. The enzyme buffer applied in this study, was already
445 proposed to detect genotoxicity by the Fpg-modified comet assay applied on fish cell lines
446 PLHC-1, RTL-W1, RTG-W1 (Kienzler et al., 2012) and on erythrocytes of Japanese Medaka,
447 *Oryzias latipes* (Pannetier et al., 2019) with concentrations of Fpg varying from 0.4 UI/gel
448 (corresponding to 0.4 U/mL) to 8 UI/ μ L diluted (corresponding to 1.6 U/mL). Other authors
449 used the same components in the buffer at different concentrations and pH [100 mM KCl,
450 10 mM EDTA, 10 mM HEPES, 0.1 mg/mL BSA, adjusted pH 7.2] for the application of the

451 Fpg-modified comet assay on erythrocytes of many fish species *Salmo cenerinus* Nardo, *Salmo*
452 *marmoratus* Cuvier, *Alburnus alburnus*, *Alburnoides bipunctatus*, *Squalius cephalus* and
453 *Alburnus alburnus* (Jovanović et al., 2018; Kolarević et al., 2018, 2016; Kračun-Kolarević et
454 al., 2016). Also, another buffer was tested [10 mM Bis-Tris propane HCl, 10 mM MgCl₂, 1
455 mM DTT, pH 7.0, 0.1 mg/mL FBS] with the Fpg concentration of 100 mU per slides
456 (corresponding to 0.1 U/mL), as proposed on erythrocytes of *Carassius auratus* to detect
457 genotoxicity from pesticide exposure (Zhao et al., 2015). For all studies, the incubation of the
458 enzyme was done at 37°C in a humidified chamber for 30 min except in the study of Zhao et
459 al. (2015), with an incubation of 45 min. Thus, the concentration of Fpg determined in our work
460 (1 U/mL) was within the range of concentrations found in the literature (0.1 U/mL to 1.6 U/mL),
461 with the most often used incubation time for freshwater fish (30 min). However, the enzyme
462 buffer used in our study on sticklebacks induced some DNA impacts, which were considered
463 to compare all net levels of Fpg sensitive sites in erythrocytes of fish corresponding to oxidative
464 and alkylating DNA damage. This checkpoint of the enzyme buffer is not always performed or
465 indicated in ecotoxicological studies. The enzyme buffer used on erythrocytes of *Salmo*
466 *cenerinus* Nardo, *Salmo marmoratus* Cuvier [100 mM KCl, 10 mM EDTA, 10 mM HEPES,
467 0.1 mg/mL BSA, adjusted pH 7.2] by Kračun-Kolarević et al. (2016) did not seem to induce
468 DNA damage. To further improve our protocol established, it could be interesting to test this
469 enzyme buffer adjustments proposed by these authors on sticklebacks' erythrocytes, while
470 keeping the concentration and incubation time of the Fpg determined in our work. This step
471 would allow to progress toward the establishment of a harmonized protocol for the Fpg-
472 modified comet assay applied on freshwater fish erythrocytes as for the comet assay in its
473 alkaline version.

474 Few studies reported the use of oxidizing agents as assay control of the enzyme-modified
475 comet assay, except for hydrogen peroxide (H₂O₂) but which mainly induces strand breaks and

476 not oxidative lesions, and mostly performed on human cells (Møller et al., 2018). Recently, the
477 photosensitizer Ro19-8022, KBrO₃ and 4-nitroquinoline-1-oxide were preconized as suitable
478 positive controls for these lesions on human mononuclear blood cells (Møller et al., 2018). The
479 Ro19-8022 plus light tested on stickleback's erythrocytes confirmed the established protocol
480 of the Fpg-modified comet assay. The digestion with Fpg enzyme on erythrocytes led to a
481 significant increase (2.7-fold) of DNA damage after the exposure to Ro19-8022 at 1 µM and
482 after 2.5 min of irradiation (1000 W-light), in comparison with the ones corresponding to
483 standard alkaline and buffer controls. This experiment demonstrated the sensibility of
484 erythrocytes to oxidative stress detected by the Fpg-modified comet assay. Notably, these
485 results are in agreement with those obtained on erythrocytes of caught wild fish where a
486 significant higher level of DNA strand breaks was highlighted after the Fpg-modified comet
487 assay in comparison with its alkaline condition. For example, the highest net Fpg contribution
488 was at 51.4 ± 1.6 TI % compared to the alkaline comet assay detecting 22.7 ± 1.3 TI % of DNA
489 strand breaks, corresponding to 2.26-fold increase of DNA lesions (Kolarević et al., 2016).

490 After the optimisation step, the application of the Fpg-modified comet assay was tested on
491 the field during an active biomonitoring study. In parallel, the effect of caging on each
492 erythrocyte biomarker was investigated as already performed for other biomarkers developed in
493 sticklebacks (Le Guernic et al., 2016b; Catteau et al., 2019). Their following recommendations
494 have been applied on the current study on the Artois-Picardie watershed, which was carried out
495 without a food intake, outside the breeding period, with contact cage sediment (when it was
496 possible) and without transport before dissection. Twenty-one days of caging was used
497 corresponding to the conventional time for fish (Oikari, 2006) and which has been previously
498 tested and shown to be effective for sticklebacks on the field studies (Catteau et al., 2020, 2021,
499 2022; Cant et al., 2022).

500 Concerning the caging impact, no significant difference was highlighted for all biomarkers
501 of genotoxicity (DNA content variation, DNA strand breaks and net Fpg-sensitive sites)
502 between “free fish” and “caged fish” from the outside pond. The same results were found on
503 the river study between the reference site at *Manqueville* (caged fish) and the control pond at
504 *Verneuil* (free fish). Besides, erythrocyte mortality was significantly higher in free living fish
505 than in caged fish from the pond (for necrosis) and from *Manqueville* (for apoptosis).
506 Xenobiotics have been shown to increase hemolysis (*i.e.* red cell breakdown) (Nikinmaa, 1992)
507 and induce eryptosis (erythrocyte programmed death) corresponding to apoptosis process, a
508 useful model for evaluating the toxic effects (Farang and Alagawany, 2018; Orrenius et al.,
509 2011). In contrast, necrosis corresponding to a traumatic and uncontrolled cell death can be
510 related to more severe conditions of exposure (Lyons-Alcantara et al., 1998; AnvariFar et al.,
511 2017). Erythrocyte mortality may provide the basis to monitor the effect of anthropogenic
512 contaminants, however reports on cell mortality in fish are still limited (AnvariFar et al., 2017).
513 For each fish from reference conditions, the erythrocyte mortality was under 20 %, which did
514 not seem to impact the genotoxic responses considered as basal levels. For example the values
515 of DNA strand breaks measured in fish from our reference sites (*Verneuil* : 8.18 ± 2.96 TI %
516 and *Manqueville* : 6.80 ± 2.38 TI %), were close to the values monitored by Santos et al. (2016)
517 in another reference station (*Houdancourt* : 5.63 ± 1.15 TI %) with wild sticklebacks, that
518 confirm the validity of our measurements. In our work, the baseline values of net Fpg-sensitive
519 sites were between 5.53 ± 3.66 and 7.64 ± 6.79 TI %, corresponding mostly to the enzyme
520 buffer impact estimated at 6.56 ± 5.78 TI % and a basal level of oxidative DNA damage in
521 cells. These results are in agreement with other studies, where net-oxo-G (net Fpg-sensitive
522 sites) were found in the same order of magnitude for less impacted stations, ranging from 0, 4.4
523 to 6 TI %, with erythrocyte viability above 70 % or on average 80 % in fish species collected
524 in rivers (Jovanović et al., 2018; Kolarević et al., 2018, 2016). The DNA content baseline of

525 sticklebacks erythrocytes varied between 0.30 ± 0.59 and 0.51 ± 0.49 CV, which were above
526 the first basal level obtained in 2019 (-0.96 ± 0.62 CV corresponding to no chromosomal
527 damage) (Cant et al., 2022) and inside the range of reference values obtained in *Scophthalmus*
528 *maximus* ($0,50 \pm 0,06$ and $0,29 \pm 0,08$ CV) (Goanvec et al., 2004). So, caging could be removed
529 from the list of potential confounding factors for the genotoxicity and mortality biomarker
530 responses in sticklebacks.

531 Concerning the reference station, no significant difference of the erythrocyte density
532 measured in *Manqueville* was found compared to free to swim sticklebacks in *Verneuil*
533 (4.28 ± 1.01 vs $6.03 \pm 3.90 \times 10^5$ cell/mm³), which were in the lowest of density found in fish
534 usually ranging from $0.5\text{--}1.5 \times 10^6/\text{mm}^3$ to $3.0\text{--}4.2 \times 10^6/\text{mm}^3$ (Witeska, 2013). The cell
535 density was significantly lower in caged fish from contaminated stations ranging from
536 2.17 ± 0.96 to $3.63 \pm 1.36 \times 10^5$ cell/mm³ in 2020, corresponding to levels obtained in stations
537 in 2019 (1.56 ± 0.67 to $3.21 \pm 0.88 \times 10^5$ cell/mm³) (Cant et al., 2022). Erythropoiesis
538 (10-20 days in adults fish) (Murad and Houston, 1992) is slower than erythrocyte breakdown,
539 which could be impacted by many factors including any toxicants causing oxidative stress and
540 facilitating the senescence of erythrocytes (Nikinmaa, 2020). This decrease of erythrocytes
541 density on stations studied does not seem to be related either to the transfer of fish on river
542 neither to the caging, since the values obtained in reference stations (*Verneuil* and *Manqueville*)
543 were comparable. So, in this field study performed on 2020, 21 days seem to be sufficient to
544 decrease erythrocytes density before their renewal, which could be linked to environmental
545 stress.

546 At the downgraded stations, DNA strand breaks, chromosomal damages and oxidative DNA
547 damage were measured after 21 days of exposure, compared to fish under reference conditions.
548 Significant DNA strand breaks have previously been observed after 14 days of caging of
549 sticklebacks (at 29.80 ± 3.61 TI % and after 28 days to 18.44 ± 2.07 TI %) in ponds

550 contaminated by uranium (Le Guernic et al., 2016a). In a previous study carried out in the same
551 watershed in autumn 2019, DNA strand break levels were highlighted after 21 days of exposure
552 and ranged from 18.94 ± 7.85 TI % to 38.78 ± 19.69 TI % (Cant et al., 2022). One week was
553 sufficient to detect significant increase of DNA content variation (1.5-fold) in *Oncorhynchus*
554 *kisutch* caged on PAH contaminated lakes (from 40 Full Pic CV in reference to 60 FPCV in
555 polluted stations) (Barbee et al., 2008). These irreversible damages were highlighted by an
556 increase in measured CVs corresponding to clastogenic or aneugenic damages (Goanvec et al.,
557 2004; Barranger et al., 2014), that can persist and be found from 14 to 30 days after exposure
558 of *Psetta maxima* fish to HAP (1.5 fold the control condition (0.5 CV) (Goanvec et al., 2008).
559 In our knowledge, the time of fish caging required to detect oxidative damage to DNA does not
560 seem to be known for other species in active biomonitoring context.

561 In this present work, erythrocyte mortality (apoptosis and necrosis) were the most
562 discriminating biomarkers, as in the previous study for erythrocyte necrosis (Cant et al., 2022).
563 Net Fpg-sensitive sites measurement was the third among the most contributing biomarkers to
564 the PCA. More precisely, after 21 days of caging on the six stations of the Artois-Picardie
565 watershed, net Fpg-sensitive sites were significantly increased in sticklebacks' erythrocytes at
566 three sites including *St Rémy du Nord*, *Biache-Saint-Vaast* and *Courrières* compared to the
567 reference station. This discrimination of station was not clearly highlighted only by the DNA
568 strand breaks, as in 2019.

569 Fish from *Courrières* had the highest net contribution of Fpg sensitive sites
570 (23.00 ± 10.93 %), characterized by DNA strand breaks, oxidative stress, biomarkers of
571 cytotoxicity and by the lowest DNA content variation similar to the reference. This station
572 where a high mortality of caged sticklebacks was observed, seemed to be the most degraded as
573 in the study of 2019. However, biomarker's responses have been measured on the surviving
574 fish, which were probably the most resistant individuals among the sticklebacks caged at

575 *Courrières*. These high oxidative DNA lesions could result from the oxidative stress, which
576 would have an impact on erythrocytes (lowest cell density and the highest erythrocytes
577 mortality). Chromosomal damages, a consequence of unrepaired primary DNA damage, may
578 have been suppressed by the erythrocyte mortality mechanisms and would no longer be visible
579 at 21 days of fish exposure (Jha, 2008; Martins and Costa, 2017; Canedo et al., 2021). This
580 could explain the lower density of erythrocytes found in this site, which could affect the quality
581 of erythrocyte, their oxygen transport capacity and consequently the good health of fish (Farg
582 and Alagawany, 2018). Fish from *Biache-Saint-Vaast* were also characterized by net Fpg-
583 sensitive site, DNA strand breaks, erythrocyte mortality and DNA content variation. *Biache-*
584 *Saint-Vaast* could have the same profile of degradation than *Courrières*, but in a less advanced
585 stage. In opposition, biomarkers responses in fish caged at *Bouchain* were the closest to
586 *Verneuil* and *Manqueville* in comparison with other stations. The net Fpg-sensitive sites
587 response was the only parameter significantly induced compared to *Manqueville*. *Bouchain* can
588 thus be considered as the less impacted station in this study. A significant increase of net Fpg-
589 sensitive site has been measured also in fish caged at *St Rémy du Nord* in comparison with the
590 two reference stations (*Manqueville* and *Verneuil*), underlining the relevance of this biomarker
591 to discriminate stations during *in situ* studies. More precisely, for the three stations showing a
592 genotoxicity potential in comparison to the reference station *Manqueville*, the levels of DNA
593 lesions detected in sticklebacks by the Fpg-modified comet assay (*St Rémy du Nord* 2.21-fold ;
594 *Biache-Saint-Vaast* 2.46-fold and *Courrières* 4.24-fold) were higher than the ones from the
595 corresponding alkaline comet assay (*St Rémy du Nord* 1.44-fold ; *Biache-Saint-Vaast* 1.84-fold
596 and *Courrières* 1,73-fold). Hence, the Fpg-modified comet assay had allowed to measure
597 additional DNA lesions (notably oxidative stress type) not characterized by the alkaline comet
598 assay, which improved the discrimination of stations and so the genotoxicity assessment. Net
599 Fpg-sensitive sites detected in these stations may be more interesting in biomonitoring studies,

600 since oxidative stress (reactive oxygen species) seems to be the major dealing with polluted
601 environment (Kolarević et al., 2016; Pellegrini et al., 2020) and one of the main mode of action
602 of substances of the priority list chemicals of the WFD (2000/60/EC) (European Commission
603 Joint Research Centre, 2018; Mitchelmore and Chipman, 1998).

604 In accordance with Jovanović et al., (2018) and Kolarević et al., (2018) no correlation
605 between the DNA strand breaks and the Fpg-sensitive sites was highlighted in our study
606 (supplementary data 8). In addition, no correlation was obtained between primary DNA damage
607 (DNA strand breaks and net Fpg sensitive sites) and DNA content variation (supplementary
608 data 8), as observed with micronucleus in erythrocyte of *Alburnus alburnus* (Jovanović et al.,
609 2018) or *Squalius cephalus* (Kolarević et al., 2018) exposed to wastewaters. In contrast, a
610 negative correlation was observed between micronuclei and DNA strand breaks assessed on
611 erythrocyte of *Gasterosteus aculeatus* sampled from different locations with exposure to
612 various of sewage-treatment effluent and agricultural discharge (Wirzinger et al., 2007). The
613 present findings therefore confirm that use of a combination of biomarkers, such as DNA strand
614 breaks, oxidative DNA damage and chromosomal damage on erythrocytes of sticklebacks, is
615 an appropriate integrated biomarker approach of genotoxicity to detect different magnitudes of
616 DNA damage. Notably, this was already preconized for the biomonitoring of complex mixture
617 of genotoxicants in rivers in *Salmo trutta fario* (Hariri et al., 2018).

618 As a whole, the results in 2020 demonstrated the sensitivity of the comet assay enhanced
619 by the Fpg-modified comet assay on sticklebacks' erythrocytes and its capability to discriminate
620 stations under different environmental pressures. This new study allowed to refine the analysis
621 of the genotoxic potential of the sites studied during the first biomonitoring campaign in the
622 same Artois-Picardie watershed in 2019. Additional genotoxic damages were identified, mainly
623 found at *Courrières*. This suggests that the net Fpg-sensitive sites may represent a useful
624 biomarker of genotoxicity in complement to the measure of DNA strand breaks and DNA

625 content variation for freshwater biomonitoring Indeed, taking into account only one biomarker
626 of genotoxicity cannot reflect the integrality of the genotoxic potential in link with the pollution
627 of water bodies. In this context, a multi-biomarkers approach of genotoxicity as tested in
628 stickleback covering the detection of various DNA lesions seems relevant in an environmental
629 risk assessment, in order to protect both ecosystems and human health under the “one health
630 approach” (Destoumieux-Garzón et al., 2018).

631 In that perspective, it seems necessary to define both reference and/or threshold values
632 for genotoxicity biomarkers measured on erythrocytes of sticklebacks. In the present study,
633 results on fish from downgraded station were compared with the ones from two different
634 reference conditions. One was constituted of free fish from a breeding pond and the other of
635 caged sticklebacks on the field from the same population. Some biomarkers were significantly
636 different only with one of these references, highlighting the difficulty to obtain strong reference
637 values. The definition of robust basal values of biomarkers is essential in the monitoring of the
638 quality of environment, as already provided in the Marine Strategy Framework Directive
639 (MSFD, 2008/56/EC).

640 Therefore, further experiments would be necessary to study the impact of biotic (*i.e.*
641 sex, age) and abiotic (*i.e.* seasonality) factors on the response of all erythrocyte biomarkers
642 developed in sticklebacks to better interpret biological responses. In this sense, reference values
643 could be defined, to propose the use of these biomarkers to environmental managers.

644

645 **5. Conclusions**

646 This first application of the Fpg-modified comet assay developed on caged three-spined
647 stickleback has proven to be a sensitive assay and has shown its ability to discriminate different
648 downgraded stations according to their ecological and chemical status in a freshwater

649 biomonitoring study. After two years of study, the data collected represent a snapshot of the
650 status of each station. The presence of genotoxic potential along the Artois-Picardie watershed
651 was detected mainly on fish caged at *Courrières* and *Biache-Saint-Vaast* characterized by
652 oxidative DNA damage, DNA strand breaks and erythrocyte mortality and also for fish caged
653 at *Artres* and *Biache-Saint-Vaast*, which presented chromosomal abnormalities.

654 This multi-biomarker approach of genotoxicity integrating the Fpg-modified comet assay
655 in stickleback is thus promising, even if this approach remains to be refined by a better
656 knowledge of the natural variability and thus the response of each biomarker, allowing to
657 propose basal values for biomonitoring studies.

658

659 **Statements & Declarations**

660 **Ethical Approval**

661 Each experiment was conducted in accordance with European directive 2010/63/UE on the
662 protection of animals used for scientific purposes at INERIS facilities (registration number E60-
663 769-02, APAFIS #20760).

664

665 **Consent to Participate**

666 Not applicable.

667

668 **Consent to Publish**

669 Not applicable.

670

671 **Author contributions**

672 Amélie Cant : Conceptualization, Investigation, Formal analysis, Data Curation, Writing –

673 Original Draft, Visualization

674 Anne Bado-Nilles : Conceptualization, Investigation, Writing Original Draft, Visualization

675 Jean-Marc Porcher : Conceptualization, Funding acquisition, Writing – Review & Editing

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683

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690

691 **Competing Interest**

692 The authors declare that they have no known competing financial interests or personal
693 relationships that could have appeared to influence the work reported in this paper.

694

695 **Availability of data and materials**

696 Not applicable.

697

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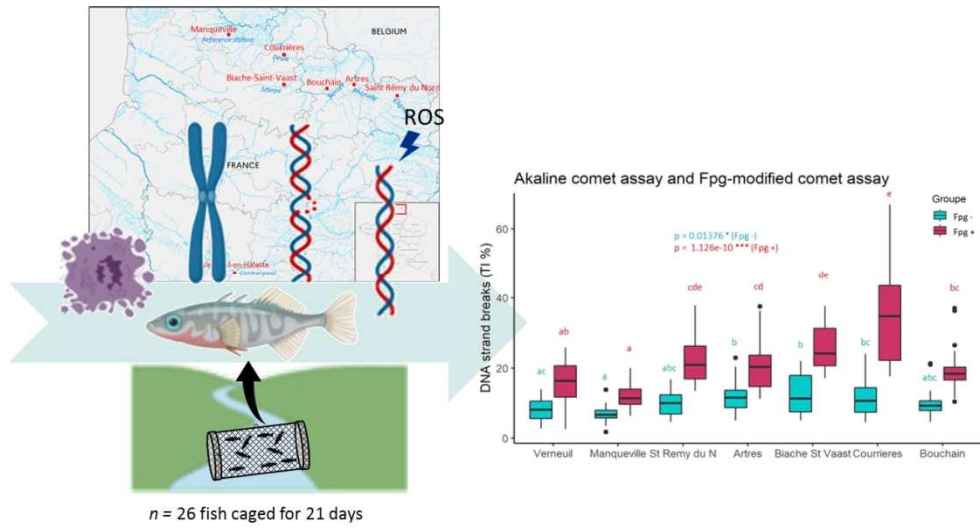
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Graphical abstract



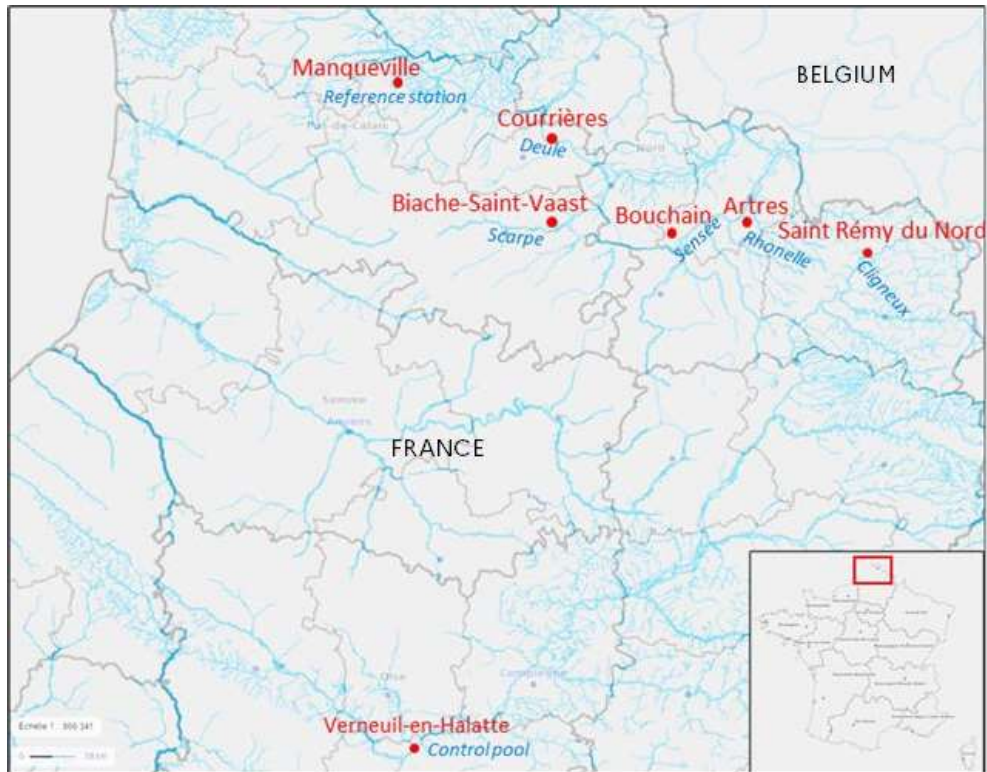


Fig. 1 Location of the control pool, the reference station and all sampling stations investigated in the Artois-Picardie watershed, north of France in autumn 2020

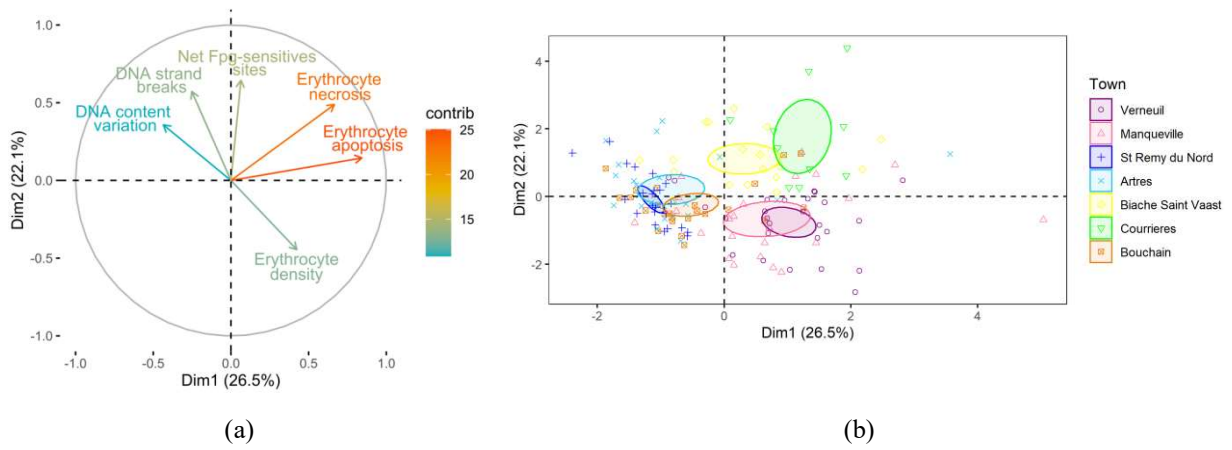


Fig. 2 Results of the principal component analysis (PCA) obtained for the MAQUEREAU 2020 project with biomarkers of erythrocyte density, mortality and genotoxicity (first and second component). PCA: (a) variables graphical and (b) individual graphical showing 95% confidence ellipses around the barycenter of the different groups. Each dot represents one fish

1 Table 1 Characteristics of sites located on the Artois-Picardie watershed: Physicochemical parameters measured were expressed with mean \pm standard deviation - Ecological
 2 and Chemical status has been collected from Water Agency of Artois-Picardie databases. <https://www.eau-artois-picardie.fr/donnees-sur-leau/visualiser-et-telecharger-les-donnees>.
 3

	Verneuil	Manqueville	St Rémy du Nord	Artres	Biache-Saint-Vaast	Courrières	Bouchain
GPS coordinates	N 49°16'21.0'' E 2°30'12.7''E	N50°34'42.9600'' E2°27'58.9320''	N 50°14'20.7'' E 3°53 '39.0''	N 50°17'50.4'' E 3°32'40.2''	N 50°18'17.3'' E 2°56'38.1''	N 50°28'08.2'' E 2°56'43.0''	N 50°16'45.4'' E 3°18'18.6''
Sandre code			1001452	1029000	1037000	1078000	1024000
WFD data	/	/	Yes	Yes	Yes	Yes	Yes
Ecological Status (2016-2018)	/	/	Bad	Mediocre	Mediocre	Mediocre	Mediocre
Chemical Status (2016-2018)	/	/	Bad	Bad	Bad	Bad	Bad
Downgrading substance	/	/	PAH	PAH	PAH	PAH and Pb	PAH
Body of surface water	Outside breeding pool	Watercress crop	Natural	Natural	Strongly modified	Artificial	Natural
Characteristic	Control pool	Reference station	Agricultural practices	Agricultural practices Artificial territory	Agricultural practices Artificial territory	Artificial territory Waterway transport	Agricultural practices Natural down stream
Temperatures (°C)	14.83 \pm 1.09	11.28 \pm 0.67	11.25 \pm 0.90	12.12 \pm 1.21	12.08 \pm 0.82	14.81 \pm 1.16	13.55 \pm 1.16
pH	8.34 \pm 0.34	7.77 \pm 0.23	8.27 \pm 0.11	8.22 \pm 0.07	7.84 \pm 0.01	8.07 \pm 0.06	7.91 \pm 0.09
Conductivity (μ S/cm)	649 \pm 50	395 \pm 40	655 \pm 20	660 \pm 110	720 \pm 30	755 \pm 40	530 \pm 10

4 PAH: polycyclic aromatic hydrocarbon. PB: lead.

5 Table 2 Synthesized results from protocols tested for the optimisation of the Fpg-modified comet assay on erythrocytes of sticklebacks, regarding (A) the incidence of enzyme
 6 buffer time incubation and (B) the Fpg concentrations. DNA strand breaks are expressed as the percentage of tail intensity (TI %) in mean \pm standard error. *n*: number of fish,
 7 Fpg: Formamidopyrimidine DNA Glycosylase and TI: Tail Intensity corresponding of mean of the two gel medians (TI %).

8

9

A: Incidence of enzyme buffer (without Fpg) on basal DNA strand breaks (TI %) incubated for 30 or 45 min at 37°C. Stars indicate statistical differences between conditions according to a Student test (paired samples, * = $p < 0.05$).

	0 min	30 min	45 min
Buffer incidence (<i>n</i> = 10)	8,37 \pm 2,59	14,41 \pm 7,98 *	15,88 \pm 8,49 *

10

11

B: Incidence of Fpg concentration on basal DNA strand breaks (TI %) at 0, 0.5 and 1 U/mL incubation for 30 min at 37°C of stickleback's erythrocytes. For DNA strand breaks, letters indicate differences between concentrations according to one-way ANOVA III and Bonferroni tests (paired sample, $p < 0.05$), *n* = 6.

	0 U/mL	0.5 U/mL	1 U/mL
Concentration incidence (<i>n</i> = 6)	10,06 \pm 2,29 ^a	16,73 \pm 5,22 ^b	18,71 \pm 6,60 ^b

12

13

14 Table 3 Results from the positive assay control (Ro19-8022) tested for the Fpg-modified comet assay on erythrocytes of sticklebacks. Fpg - : the alkaline comet assay without
 15 Fpg; Fpg + : the Fpg-modified comet assay with the protocol conditions fixed at 1 U/mL, 37°C, 30 min; Ro19-8022 + : erythrocytes of sticklebacks were exposed to 1µM of
 16 Ro19-8022 and light + : irradiation of blood cells for 2.5 or 5 min on ice under 1000 W-light. DNA strand breaks are expressed as the percentage of tail intensity (TI %) in
 17 mean ± standard error. n: number of fish, Fpg: Formamidopyrimidine DNA Glycosylase and TI: Tail Intensity corresponding of mean of the two gel medians (TI %). Data are
 18 expressed as mean ± standard deviation and stars/letters indicate differences between concentrations according to one-way ANOVA III followed by Bonferroni tests (paired
 19 sample, $p < 0.05$), $n = 4$.
 20

Condition ($n = 4$)	Fpg -	Fpg +	Fpg + Ro19-8022 +	Fpg + Ro19-8022 + 2.5 of light +	Fpg + Ro19-8022 + 5 of light +
DNA strand breaks (TI %)	12.13 ± 3.19 ^a	20.22 ± 4.06 ^{ab}	17.44 ± 2.43 ^a	47.78 ± 2.05 ^b (x 2.7)*	62.52 ± 10.68 ^b (x 3.6)*
Hedgehogs	3.00 ± 3.56	3.25 ± 1.71	5.12 ± 3.38	8.83 ± 3.55	4.12 ± 1.38

21 *The fold increase is calculated in comparison with the “Fpg + Ro19-8022+” condition.
 22

23 Table 4 Biometric index and genotoxic biomarker responses assessed on sticklebacks erythrocytes on fish caged for 21 days or free to swim in the pool (Vermeuil-en-Halatte,
 24 October 2021). Data are expressed as mean \pm standard deviation and stars indicate statistical differences between “Free fish” and “Caging fish” according to a Student or
 25 Wilcoxon-Mann-Whitney test (independent sample, *** = $p < 0.001$).
 26

	Free fish ($n = 30$)		Caged fish ($n = 29$)	
	Mean	Sd	Mean	Sd
Biometric index				
Standard length (mm)	48.77 \pm 5.35	***	44.17 \pm 2.18	
Weight (g)	1.87 \pm 0.68	***	1.07 \pm 0.18	
Erythrocyte density	Fresh erythrocyte (cell/mm ³ x 10 ⁵)	4.28 \pm 1.01	***	2.18 \pm 1.55
Erythrocyte mortality	Erythrocyte necrosis (%)	13.87 \pm 8.00	***	5.68 \pm 5.07
	Erythrocyte apoptosis (%)	6.35 \pm 7.53		7.31 \pm 10.29
Chromosomal damage	DNA content variation (CV)	1.69 \pm 1.13		1.78 \pm 1.52
Alkaline comet assay	DNA strand breaks (TI %)	8.37 \pm 3.91		9.38 \pm 4.58
	Hedgehogs (%)	1.47 \pm 2.26		1.83 \pm 2.05
Fpg-modified comet assay	DNA strand breaks (TI %)	16.03 \pm 6.28		15.92 \pm 6.49
	Hedgehogs (%)	4.03 \pm 5.48		4.78 \pm 6.29
	Net Fpg-sensitive sites (TI %)	7.67 \pm 4.71		6.54 \pm 5.77

27 Free fish enzyme buffer-alkaline comet assay (DNA strands breaks, $n = 10$) = 7.67 \pm 7.55 TI % (mean of medians)
 28 Caged fish enzyme buffer-alkaline comet assay (DNA strands breaks, $n = 10$) = 3.92 \pm 4.06 TI % (mean of medians)
 29 In mean the buffer impact was estimated of 6.56 \pm 5.78 TI % on these mixed groups of caged and free fish.
 30

32 Table 5 Biometric index, erythrocyte density, erythrocyte mortality and genotoxicity biomarker responses assessed on sticklebacks erythrocytes after 21 days of caging in the
 33 6 stations studied of the Artois-Picardie in October 2020 and in a control pool at Verneuil. Data are expressed as mean \pm standard deviation and letters represent significant
 34 differences between stations according to two-way ANOVA III or Kruskal-Wallis tests following by their corresponding post hoc analysis. (independent samples, $p < 0.05$).

35

	October 2020	Sex*	Verneuil	Manqueville	St Rémy du Nord	Artres	Biache-Saint-Vaast	Courrières	Bouchain
Biometric index	<i>N</i>	M	6	5	10	6	4	3	6
		F	20	18	13	19	14	7	18
Standard length (mm)	M/F	46.19 \pm 3.02	45.09 \pm 1.78	45.00 \pm 2.88	45.08 \pm 2.94	45.22 \pm 2.80	44.90 \pm 2.96	44.04 \pm 3.63	
Weight (g)	M/F	1.21 \pm 0.24	1.38 \pm 0.22	1.13 \pm 0.23	1.17 \pm 0.21	1.23 \pm 0.25	1.17 \pm 0.25	1.09 \pm 0.29	
Erythrocyte density	M/F	6.03 \pm 3.90 ^c	4.71 \pm 3.13 ^{bc}	2.72 \pm 0.53 ^{ab}	3.25 \pm 0.72 ^{ab}	3.63 \pm 1.36 ^{bc}	2.17 \pm 0.96 ^a	2.80 \pm 0.94 ^{ab}	
Erythrocyte mortality	M/F	11.53 \pm 9.58 ^c	14.91 \pm 11.62 ^{cd}	1.01 \pm 0.59 ^a	6.20 \pm 8.39 ^b	20.64 \pm 8.13 ^d	20.10 \pm 8.85 ^d	5.03 \pm 5.22 ^b	
Chromosomal damage	M/F	7.16 \pm 4.16 ^d	4.91 \pm 6.88 ^{bc}	0.45 \pm 0.67 ^a	2.17 \pm 5.11 ^{ab}	4.51 \pm 4.51 ^{cd}	9.45 \pm 4.87 ^d	2.23 \pm 3.12 ^{bc}	
Alkaline comet assay	M/F	0.30 \pm 0.59 ^a	0.51 \pm 0.49 ^{abc}	0.86 \pm 0.88 ^{bcd}	1.14 \pm 0.62 ^d	1.06 \pm 0.55 ^{dc}	0.19 \pm 0.60 ^a	0.44 \pm 0.66 ^{ab}	
Fpg-modified comet assay	M/F	8.18 \pm 2.96 ^{ac}	6.80 \pm 2.38 ^a	9.81 \pm 3.58 ^{abc}	11.87 \pm 4.52 ^b	12.48 \pm 5.73 ^b	11.80 \pm 6.08 ^{bc}	10.06 \pm 4.15 ^{abc}	
	M/F	NA	0.85 \pm 0.95	1.11 \pm 1.23	4.18 \pm 4.58	1.50 \pm 1.73	1.00 \pm 1.75	0.89 \pm 1.19	
	M/F	15.72 \pm 5.93 ^{ab}	12.27 \pm 3.72 ^a	22.02 \pm 6.62 ^{cde}	20.93 \pm 6.95 ^{cd}	25.72 \pm 6.37 ^{de}	35.23 \pm 15.51 ^e	19.47 \pm 6.59 ^{bc}	
	M/F	NA	4.30 \pm 4.05	2.52 \pm 2.86	7.96 \pm 13.16	5.09 \pm 8.07	9.50 \pm 19.65	4.56 \pm 6.70	
	M/F	7.64 \pm 6.79 ^{ab}	5.53 \pm 3.66 ^a	12.21 \pm 5.56 ^b	9.07 \pm 5.98 ^{ab}	13.60 \pm 5.03 ^b	23.43 \pm 10.93 ^c	9.47 \pm 7.59 ^{ab}	

36 * Data M/F indicates no significant impact of sex on any of parameters studied.

37 Table 6 Biometric index (Fulton's condition, weight, standard condition) expressed in mean \pm standard deviation for each station. Stars represent a significant difference
 38 obtained between the biometric index at T0 (the first day of the caging) and at T21 (after 21 days of caging in the field) according to a Student or Wilcoxon-Mann-Whitney
 39 test (independent samples, *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$). One sampling was carried out for fish from Verneuil, of which here are the data obtained for
 40 Standard length = 46.19 ± 3.02 mm, Weight = 1.21 ± 0.24 g and Fulton's condition = 1.22 ± 0.17 .

	Standard length T0 (mm)		Standard length T21 (mm)		Weight T0 (g)		Weight T21 (g)		Fulton's condition index T0		Fulton's condition index T21	
	Mean \pm Sd	Mean \pm Sd	Mean \pm Sd	Mean \pm Sd	Mean \pm Sd	Mean \pm Sd	Mean \pm Sd	Mean \pm Sd	Mean \pm Sd	Mean \pm Sd	Mean \pm Sd	
Manqueville	45.04 \pm 1.78	45.09 \pm 1.78	1.30 \pm 0.18	1.38 \pm 0.22	1.30 \pm 0.18	1.38 \pm 0.22	1.41 \pm 0.13	1.50 \pm 0.15	1.41 \pm 0.13	1.50 \pm 0.15	1.41 \pm 0.13	1.50 \pm 0.15 *
St Rémy du Nord	44.85 \pm 2.49	45.00 \pm 2.88	1.36 \pm 0.27	1.13 \pm 0.23 **	1.36 \pm 0.27	1.13 \pm 0.23 **	1.50 \pm 0.18	1.24 \pm 0.17 ***	1.50 \pm 0.18	1.24 \pm 0.17 ***	1.50 \pm 0.18	1.24 \pm 0.17 ***
Artres	45.58 \pm 2.76	45.08 \pm 2.94	1.32 \pm 0.24	1.18 \pm 0.21 *	1.32 \pm 0.24	1.18 \pm 0.21 *	1.40 \pm 0.16	1.27 \pm 0.13 **	1.40 \pm 0.16	1.27 \pm 0.13 **	1.40 \pm 0.16	1.27 \pm 0.13 **
Biache-Saint-Vaast	45.15 \pm 2.96	45.22 \pm 2.80	1.32 \pm 0.29	1.23 \pm 0.26	1.32 \pm 0.29	1.23 \pm 0.26	1.42 \pm 0.18	1.32 \pm 0.13 *	1.42 \pm 0.18	1.32 \pm 0.13 *	1.42 \pm 0.18	1.32 \pm 0.13 *
Courrières	44.00 \pm 2.76	44.90 \pm 2.96	1.15 \pm 0.22	1.17 \pm 0.25	1.15 \pm 0.22	1.17 \pm 0.25	1.34 \pm 0.14	1.28 \pm 0.15	1.34 \pm 0.14	1.28 \pm 0.15	1.34 \pm 0.14	1.28 \pm 0.15
Bouchain	44.38 \pm 3.51	44.04 \pm 3.63	1.19 \pm 0.28	1.08 \pm 0.30	1.19 \pm 0.28	1.08 \pm 0.30	1.32 \pm 0.11	1.24 \pm 0.09 **	1.32 \pm 0.11	1.24 \pm 0.09 **	1.32 \pm 0.11	1.24 \pm 0.09 **