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

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19 Abstract

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

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

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39 Highlights:

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

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

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

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

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

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

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

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

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

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

2. Materials and Method 142

2.1. Fish blood sampling 143

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

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

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2.2. Optimisation of the FPG-modified comet assay on peripherical erythrocytes

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

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

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

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

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

In September/October 2020, 156 one-year-old sticklebacks with homogeneous length 216 $(44.83 \pm 0.57 \text{ mm})$ and weight $(1.27 \pm 0.08 \text{ g})$ were sexed (De Kermoysan et al., 2013) and 217 caged for 21 days by batch of 26 fish in a 36 L cylindrical stainless-steel tanks in six distinct 218 stations in the north of France (Fig. 1). These caging conditions (fish density and no external 219 220 food supply) were recommended as inducing no effect on biomarkers responses (Le Guernic et al., 2016b; Catteau et al., 2019). The Cligneux river at Saint-Rémy du Nord ("St Rémy du Nord 221 "station), the Rhonelle river at Artres ("Artres" station,) the Scarpe river at Biache-Saint-Vaast 222 ("Biache-Saint-Vaast" station), the Deule river at Courrières ("Courrières" station and the 223 Sensée river at Bouchain ("Bouchain" station) were previously described in Cant et al. (2022). 224 All these sites are subjected to various anthropogenic pressures and contaminated by polycyclic 225 aromatic hydrocarbons (PAHs), considered as downgrading substances (WFD, 2000/60/CE) 226 (Agence de l'Eau Artois-Picardie 2020). A watercress crop at Manqueville ("Manqueville" 227 228 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 other biomarkers measured in sticklebacks (Catteau et al., 2019). In this sense, a different batch 232 of fish (October 2021) was divided into two groups of 30 specimens (30 free and 29 caged fish 233 collected after 21 days) from the same outside breeding pond. 234

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In each station, temperature was monitored continuously using a probe hooked to the cage (HOBO Pendant Temperature Data Logger, Onset, Bourne, MA, USA). Water, pH and conductivity were measured at the first day and last day of exposure in each river by using a WTW multi-parameter instrument equipped with digital sensors (MultiLine® IDS, Multi3430, 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 $\pm 40 \ \mu$ S/cm) and the highest in *Courrières* (755 $\pm 40 \ \mu$ S/cm). These physico-244 chemical parameters were detailed in Table 1.

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

Chromosomal damage was determined on erythrocytes according to the original method developed by Vindeløv and Christensen (1990) and adapted by Marchand et al. (2017) on stickleback. Erythrocytes densities were adjusted at 40.10⁶ cells/mL. Successive treatments were applied to disrupt cytoplasmic and nuclear membranes to disrupt RNA and access DNA, stabilize amino acids and mark DNA by the propidium iodide (1 mg/mL in water, Invitrogen, Carlsbad, CA, United States). A sample of stabilized chicken red blood cells (Fitzgerald Industries, Acton, United States) was used as a standard and analyzed simultaneously. Each
PerCP-Vio700-A peak coefficient of variation (CV) corresponds to DNA content variation,
referred to chromosomal aberrations. DNA damage corresponds to the CV of fish erythrocyte
samples minus the CV of chicken red blood cell samples (Easton et al., 1997). All samples were
analyzed by FCM (30 000 events) (MacsquantX, Miltenyi Biotec, USA).

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272 **2.4.** Statistical analysis

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

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

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

Finally, a standardized principal component analysis (PCA) was performed to illustrate the discrimination of stations with all biomarker responses on the two first principal dimensions (package *FactoMine R*). All hypotheses were tested at the level of $p \le 0.05$. The statistical analyses are detailed in the Tables Supplementary data 1 to 8. 299 **3. Results**

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

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

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

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

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

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

Caging of fish did not induce significant effect on erythrocyte apoptosis $(7.31 \pm 10.29 \%)$, 334 *p*-value = 0.89), Fpg-sensitive sites (6.54 ± 5.77 TI %, *p*-value = 0.43), DNA strand breaks 335 $(9.38 \pm 4.58 \text{ TI }\%, p$ -value = 0.47) and DNA content variation $(1.78 \pm 1.52 \text{ CV}, p$ -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 $(2.18 \times 10^5 \pm 1.55 \text{ cell} / \text{mm}^3, p$ -value = $1.13e^{-08})$ and necrosis 339 $(5.68 \pm 5.07 \%, p$ -value = 2.97e⁻⁰⁶) of erythrocytes were significantly lower in caged fish 340 $(4.28 \times 10^5 \pm 1.01 \text{ cell} / \text{mm}^3, 13.87 \pm 8.00 \%$, respectively; Table 4, Supp. data 4). 341

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

The number of sticklebacks collected after 21 days of exposure and all responses of biomarkers for each group of fish in each station are shown in Table 5 (mean \pm sd). These raw values were used for all the analyses presented below. Out of 123 caged sticklebacks were collected from the field, with 27.64 % of males and 72.36 % of females. No significant impact
of gender was demonstrated on any of the parameters studied (*p*-values > 0.05, Supp. data 5).
The survival rate of fish was 96.15 % in *Artres*, 92.31 % in *Bouchain*, 88.46 % in *Manqueville*as in *St Rémy du Nord*, 69.23 % in *Biache-Saint-Vaast* and 38.46 % in *Courrières*, where a high
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 principal components of the PCA (Fig. 2, Supp. data 6) was explained in the first axis (26.5 %) 354 especially by erythrocyte apoptosis (44.66 % of contribution) and necrosis (27.89 % of 355 contribution), DNA content variation (11.90 % of contribution) and erythrocyte density 356 (11.30 % of contribution) and in the second axis (22.1 %) mainly due to net Fpg-sensitive sites 357 (31.26 % of contribution), DNA strand breaks (24.66 % of contribution), erythrocyte necrosis 358 (17.95 % of contribution) and fresh erythrocyte (14.93 % of contribution) (Fig. 2, Supp. data 6). 359 The first group of the PCA concern reference stations (Verneuil, Manqueville) (Fig. 2, Table 360 5). This reference group was characterised by the highest erythrocyte densities $(6.03 \pm 3.90 \text{ x})$ 361 10^5 cell/mm³ at *Verneuil* and $4.71 \pm 3.13 \times 10^5$ cell/mm³ at *Mangueville*) associated with an 362 erythrocyte mortality characterised by a strong inter-individual variability (Verneuil: 363 11.53 ± 9.58 % for necrosis and 7.16 ± 4.16 % for apoptosis; *Manqueville*: 14.91 ± 11.62 % for 364 necrosis and 4.91 ± 6.68 % for apoptosis). Overall, the genotoxic parameters were lower than 365 in other groups (DNA strand breaks: 8.18 ± 2.96 TI % at Verneuil, 6.80 ± 2.38 TI % at 366 Manqueville; Fpg-sensitive sites: 7.64 \pm 6.79 TI % at Verneuil, 5.53 \pm 3.66 TI % at 367 Manqueville; DNA content variation: 0.30 ± 0.59 CV at Verneuil, 0.51 ± 0.49 CV at 368 Manqueville). Manqueville was also characterised by a significant increase of Fulton's 369 condition index observed after the exposure (from 1.41 ± 0.13 to 1.50 ± 0.15 , *p*-values = 0.042, 370 371 Table 6, Supp. data 7) and a significant decrease of erythrocyte apoptosis in caged fish at

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

The two other groups concern fish from the downgraded stations. Except at Courrières 374 corresponding to the station characterised by the highest fish mortality, a significant decrease 375 of the Fulton's condition index (K) was shown after 21 days of caging (St Rémy du Nord : from 376 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*-377 values = $4,69e^{-3}$; *Biache-Saint-Vaast*: from 1.42 ± 0.18 to 1.32 ± 013 , *p*-values = $1.92e^{-2}$; 378 Bouchain : from 1.32 ± 0.11 to 1.24 ± 0.09 , p-values = $6.53e^{-3}$), which could be due to weight-379 loss as significantly observed at St Rémy du Nord (16.91 % of decreasing, p-values =2.64e⁻³) 380 and Artres (10.61 % of decreasing, p-values = $2.35e^{-2}$). More specifically, a second group 381 gathered the fish caged at Biache-Saint-Vaast and Courrières (Fig. 2, Table 5). These 382 sticklebacks were mainly characterised by high data of erythrocyte mortality (Biache-Saint-383 *Vaast*: 20.64 ± 8.13 % for necrosis and 4.51 ± 4.51 % for apoptosis; *Courrières*: 20.10 ± 8.85 % 384 for necrosis and 9.45 ± 4.87 % for apoptosis), by a trend towards to increase for DNA strand 385 breaks (12.48 \pm 5.73 TI % at *Biache-Saint Vaast*; 11.80 \pm 6.08 TI % at *Courrières*) and by high 386 net Fpg-sensitive sites (13.60 \pm 5.03 TI % at *Biache-Saint-Vaast* and 23.43 \pm 10.93 TI % at 387 *Courrières*). This group was also characterised by low erythrocyte density $(3.63 \pm 1.36 \times 10^5)$ 388 cell/mm³ at *Biache-Saint-Vaast* and $2.17 \pm 0.96 \text{ x} 10^5 \text{ cell/mm}^3$ at *Courrières*). 389

A last group formed by the fish caged at *St Rémy du Nord*, *Artres* and *Bouchain* was also characterised by lower values whatever the biomarker considered (erythrocyte density : $2.72 \pm 0.53 \times 10^5$ cell/mm³ at *St Rémy du Nord*, $3.25 \pm 0.72 \times 10^5$ cell/mm³ at *Artres* and $3.63 \pm 1.36 \times 10^5$ cell/mm³ at *Bouchain*; necrosis : 1.01 ± 0.59 % at *St Rémy du Nord*, 6.20 ± 8.39 % at *Artres*, 5.03 ± 5.22 % at *Bouchain*; apoptosis : 0.45 ± 0.67 % at *St Rémy du Nord*, 2.17 ± 5.11 % at *Artres*, 2.23 ± 3.12 % at *Bouchain*; DNA strand breaks : 9.81 ± 3.58 TI % at *St Rémy du Nord*, 11.87 ± 4.52 TI % at *Artres*, 10.06 ± 4.15 TI % at Bouchain; net Fpg-sensitive sites : 12.21 ± 5.56 TI % at *St Rémy du Nord*, 9.07 ± 5.98 TI % at *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 Verneuil and in the reference station Mangueville (11.87 \pm 4.52 TI %, 12.48 \pm 5.73 TI %, 401 8.18 ± 2.96 TI % and 6.80 ± 2.38 TI % respectively, *p*-values = $1.13e^{-2}$, Table 5, Supp. data 5), 402 403 whereas additional DNA damage have been highlighted with the inclusion of Fpg. The latter 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 405 406 assessed in fish from Verneuil and Manqueville (22.02 \pm 6.62 TI %, 20.93 \pm 6.95 TI %, 25.72 ± 6.37 TI %, 35.23 ± 15.51 TI %, 15.72 ± 5.93 TI %, 12.27 ± 3.72 TI %; respectively, 407 *p*-values = 1.12e-10, Table 5, Supp. data 5). The discrimination of station was improved by the 408 integration of DNA strand breaks measured with Fpg compared to the levels obtained without 409 Fpg (Table 5). Indeed, fish caged in Courrières were characterized by a significant rise of net 410 411 Fpg-sensitive sites $(23.43 \pm 10.93 \text{ TI }\%)$ compared to the two reference stations $(7.64 \pm 6.79 \text{ TI }\%)$ % at *Verneuil* and 5.53 ± 3.66 TI % at *Manqueville*, *p*-values = 4.45e-07, Table 5, Supp. data 5). 412 A significant induction of net Fpg-sensitive sites was also found in fish from St Rémy du Nord 413 414 and *Biache-Saint-Vaast* but only compared to *Manqueville* $(12.21 \pm 5.56 \% \text{ TI}, 13.60 \pm 5.03 \%$ TI, 5.53 ± 3.66 TI %, respectively, *p*-values = 4.45e-07, Table 5, Supp. data 5). 415

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

426 **4. Discussion**

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

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

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

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

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

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

Concerning the caging impact, no significant difference was highlighted for all biomarkers 500 of genotoxicity (DNA content variation, DNA strand breaks and net Fpg-sensitive sites) 501 between "free fish" and "caged fish" from the outside pond. The same results were found on 502 the river study between the reference site at Manqueville (caged fish) and the control pond at 503 Verneuil (free fish). Besides, erythrocyte mortality was significantly higher in free living fish 504 than in caged fish from the pond (for necrosis) and from Mangueville (for apoptosis). 505 Xenobiotics have been shown to increase hemolysis (*i.e.* red cell breakdown) (Nikinmaa, 1992) 506 507 and induce eryptosis (erythrocyte programmed death) corresponding to apoptosis process, a useful model for evaluating the toxic effects (Farag and Alagawany, 2018; Orrenius et al., 508 2011). In contrast, necrosis corresponding to a traumatic and uncontrolled cell death can be 509 related to more severe conditions of exposure (Lyons-Alcantara et al., 1998; AnvariFar et al., 510 2017). Erythrocyte mortality may provide the basis to monitor the effect of anthropogenic 511 512 contaminants, however reports on cell mortality in fish are still limited (AnvariFar et al., 2017). For each fish from reference conditions, the erythrocyte mortality was under 20 %, which did 513 514 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 % and *Manqueville* : 6.80 ± 2.38 TI %), were close to the values monitored by Santos et al. (2016) 516 in another reference station (Houdancourt : 5.63 ± 1.15 TI %) with wild sticklebacks, that 517 confirm the validity of our measurements. In our work, the baseline values of net Fpg-sensitive 518 sites were between 5.53 \pm 3.66 and 7.64 \pm 6.79 TI %, corresponding mostly to the enzyme 519 buffer impact estimated at 6.56 ± 5.78 TI % and a basal level of oxidative DNA damage in 520 521 cells. These results are in agreement with other studies, where net-oxo-G (net Fpg-sensitive sites) were found in the same order of magnitude for less impacted stations, ranging from 0, 4.4 522 to 6 TI %, with erythrocyte viability above 70 % or on average 80 % in fish species collected 523 in rivers (Jovanović et al., 2018; Kolarević et al., 2018, 2016). The DNA content baseline of 524

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

531 Concerning the reference station, no significant difference of the erythrocyte density measured in Manqueville was found compared to free to swim sticklebacks in Verneuil 532 $(4.28 \pm 1.01 \text{ vs } 6.03 \pm 3.90 \text{ x } 10^5 \text{ cell/mm}^3)$, which were in the lowest of density found in fish 533 usually ranging from $0.5-1.5 \times 10^{6}$ /mm³ to $3.0-4.2 \times 10^{6}$ /mm³ (Witeska, 2013). The cell 534 density was significantly lower in caged fish from contaminated stations ranging from 535 2.17 ± 0.96 to 3.63 ± 1.36 x 10^5 cell/mm³ in 2020, corresponding to levels obtained in stations 536 in 2019 (1.56 \pm 0.67 to 3.21 \pm 0.88 10⁵ x cell/mm³) (Cant et al., 2022). Erythropoiesis 537 (10-20 days in adults fish) (Murad and Houston, 1992) is slower than erythrocyte breakdown, 538 539 which could be impacted by many factors including any toxicants causing oxidative stress and facilitating the senescence of erythrocytes (Nikinmaa, 2020). This decrease of erythrocytes 540 density on stations studied does not seem to be related either to the transfer of fish on river 541 neither to the caging, since the values obtained in reference stations (Verneuil and Manqueville) 542 were comparable. So, in this field study performed on 2020, 21 days seem to be sufficient to 543 decrease erythrocytes density before their renewal, which could be linked to environmental 544 stress. 545

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

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

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

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

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

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

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

As a whole, the results in 2020 demonstrated the sensitivity of the comet assay enhanced by the Fpg-modified comet assay on sticklebacks' erythrocytes and its capability to discriminate stations under different environmental pressures. This new study allowed to refine the analysis of the genotoxic potential of the sites studied during the first biomonitoring campaign in the same Artois-Picardie watershed in 2019. Additional genotoxic damages were identified, mainly found at *Courrières*. This suggests that the net Fpg-sensitive sites may represent a useful 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 for genotoxicity biomarkers measured on erythrocytes of sticklebacks. In the present study, 632 633 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 caged sticklebacks on the field from the same population. Some biomarkers were significantly 635 different only with one of these references, highlighting the difficulty to obtain strong reference 636 values. The definition of robust basal values of biomarkers is essential in the monitoring of the 637 quality of environment, as already provided in the Marine Strategy Framework Directive 638 (MSFD, 2008/56/EC). 639

Therefore, further experiments would be necessary to study the impact of biotic (*i.e.* sex, age) and abiotic (*i.e.* seasonality) factors on the response of all erythrocyte biomarkers developed in sticklebacks to better interpret biological responses. In this sense, reference values 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 biomonitoring study. After two years of study, the data collected represent a snapshot of the status of each station. The presence of genotoxic potential along the Artois-Picardie watershed was detected mainly on fish caged at *Courrières* and *Biache-Saint-Vaast* characterized by oxidative DNA damage, DNA strand breaks and erythrocyte mortality and also for fish caged at *Artres* and *Biache-Saint-Vaast*, which presented chromosomal abnormalities.

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

659 Statements & Declarations

769-02, APAFIS #20760).

660 Ethical Approval

- Each experiment was conducted in accordance with European directive 2010/63/UE on the protection of animals used for scientific purposes at INERIS facilities (registration number E60-
- 664

663

- 665 Consent to Participate
- 666 Not applicable.
- 667
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670

671 Author contributions

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690

691 Competing Interest

692 The authors declare that they have no known competing financial interests or personal693 relationships that could have appeared to influence the work reported in this paper.

694

695 Availability of data and materials

696 Not applicable.

698 References

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Graphical abstract



n = 26 fish caged for 21 days



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

les-donnees.							
	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 WFD data	~	1	1001452 Yes	1029000 Yes	1037000 Yes	1078000 Yes	1024000 Yes
Ecological Status (2016-2018)		~	Bad	Mediocre	Mediocre	Mediocre	Mediocre
Chemical Status (2016-2018)		1	Bad	Bad	Bad	Bad	Bad
Downgrading substance	/	/	РАН	РАН	РАН	PAH and Pb	РАН
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 ± 1.09	11.28 ± 0.67	11.25 ± 0.90	12.12 ± 1.21	12.08 ± 0.82	14.81 ± 1.16	13.55 ± 1.16
Hq	8.34 ± 0.34	7.77 ± 0.23	8.27 ± 0.11	8.22 ± 0.07	7.84 ± 0.01	8.07 ± 0.06	7.91 ± 0.09
Conductivity (µS/cm)	649 ± 50	395 ± 40	655 ± 20	660 ± 110	720 ± 30	755 ± 40	530 ± 10
PAH: polycyclic aron	atic hydrocarbon. PB: l	lead.					

Table 1 Characteristics of sites located on the Artois-Picardie watershed: Physicochemical parameters measured were expressed with mean ± standard deviation - Ecological

Table

Fpg: Formamidopyrimidine DNA Glycosylase and TI: Tail A: Incidence of enzyme buffer (without Fpg) on be conditions according to a Student test (naired sample	Intensity corresponding of mean of th sal DNA strand breaks (TI %) incubated s $* = n < 0.05$)	ne two gel medians (TI %). for 30 or 45 min at 37°C. Stars indice	ate statistical differences between
	0 min	30 min	45 min
Buffer incidence	8.37 ± 2.59	$14.41 \pm 7.98 *$	
(n = 10)	9.82 ± 5.85		15.88 ± 8.49 *
B: Incidence of Fpg concentration on basal DNA strand breaks (7	[1 %) at 0, 0.5 and 1 U/mL incubation for	30 min at 37°C of stickleback's erythro	ocytes. For DNA strand breaks, letters indicate
differences between concentrations according to one-way ANOV	A III and Bonferroni tests (paired sample,	p = < 0.05, $n = 6$.	
	0 U/mL	0.5 U/mL	1 U/mL
Concentration incidence			

 $18,71\pm6,60^{\rm b}$

 $16,73\pm5,22^{\rm b}$

 $10,06\pm2,29^{\mathrm{a}}$

(9 = u)

Table 2 Synthetized results from protocols tested for the optimisation of the Fpg-modified comet assay on erythrocytes of sticklebacks, regarding (A) the incidence of enzyme buffer time incubation and (B) the Fpg concentrations. DNA strand breaks are expressed as the percentage of tail intensity (T1 %) in mean \pm standard error. *n*: number of fish, Fpg:

0 N 8 0

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 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 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 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 expressed as mean ± standard deviation and stars/letters indicate differences between concentrations according to one-way ANOVA III followed by Bonferroni tests (paired sample, p = < 0.05, n = 4. 14 15 115 117 118 118 20

Condition			- 22 12	Fpg +	Fpg +
CORDINOII	Fpg –	Fpg +	rpg+ Dolo oooo	Ro19-8022 +	Ro19-8022 +
$(\mathbf{t} - \mathbf{t})$			H017-0022 +	2.5 of light +	5 of light +
DNIA strand breaks (TI 0%)	$17 13 \pm 3 10^{a}$	30.77 ± 4.06 ab	$17 AA + 7 A3^{a}$	$47.78 \pm 2.05^{\rm b}$	$62.52\pm10.68^{\rm b}$
DIVES SUBILI DI CANS (11 /0)	$C1.C \pm C1.71$	00.4 + 77.02	C+.7	(x 2.7)*	(x 3.6)*
Hedgehogs	3.00 ± 3.56	3.25 ± 1.71	5.12 ± 3.38	8.83 ± 3.55	4.12 ± 1.38

*The fold increase is calculated in comparison with the "Fpg + Ro19-8022+" condition.

October 2021). Data are expressed as mean ± standard deviation and stars indicate statistical differences between "Free fish" and "Caging fish" according to a Student or 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 (Verneuil-en-Halatte, Wilcoxon-Mann-Whitney test (independent sample, *** = p < 0.001).

		Fre	e fish			Cag	ed f	ĭsh
		: <i>u</i>)	= 30)			u)	7 =	y)
		Mean	S	р		Mean		Sd
Diamatuia indau	Standard length (mm)	48.77	± 5.	35 *	* *	44.17	H	2.18
DIOMETIC INDEX	Weight (g)	1.87	± 0.0	68 *	*	1.07	H	0.18
Erythrocyte density	Fresh erythrocyte (cell/mm ³ x 10 ⁵)	4.28	+ 1.	01 *	* *	2.18	++	1.55
للسطامين متصطما أغبر	Erythrocyte necrosis (%)	13.87	± 8.	* 00	* *	5.68	н	5.07
Eryunocyte mortanty	Erythrocyte apoptosis (%)	6.35	± 7.	53		7.31	H	10.29
Chromosomal damage	DNA content variation (CV)	1.69	± 1.	13		1.78	H	1.52
Alkaline comet assay	DNA strand breaks (TI %)	8.37	± 3.	91		9.38	н	4.58
	Hedgehogs (%)	1.47	± 2.	26		1.83	H	2.05
	DNA strand breaks (TI %)	16.03	± 6.3	28		15.92	Ŧ	6.49
Fpg-modified comet assay	Hedgehogs (%)	4.03	± 5.	48		4.78	н	6.29
	Net Fpg-sensitive sites (TI %)	7.67	++	71		6.54	++	5.77
		;						

Free fish enzyme buffer-alkaline comet assay (DNA strands breaks, n = 10) = 7.67 ± 7.55 TI % (mean of medians)

Caged fish enzyme buffer-alkaline comet assay (DNA strands breaks, n = 10) = 3.92 ± 4.06 Tl % (mean of medians) 27 28 29 30

In mean the buffer impact was estimated of 6.56 ± 5.78 TI % on these mixed groups of caged and free fish.

Table 5 Biometric index, erythrocyte density, erythrocyte mortality and genotoxicity biomarker responses assessed on sticklebacks erythrocytes after 21 days of caging in the 6 stations studied of the Artois-Picardie in October 2020 and in a control pool at Verneuil. Data are expressed as mean ± standard deviation and letters represent significant 32 33

35		- (m				and Summado		d (and the second	
	October 2020	Sex*	Verneuil	Manqueville	St Rémy du Nord	Artres	Biache-Saint- Vaast	Courrières	Bouchain
	Ĩ	М	9	5	10	9	4	ю	6
	N	Ч	20	18	13	19	14	7	18
Biometric inde.	x Standard length (mm)	M/F	46.19 ± 3.02	45.09 ± 1.78	45.00 ± 2.88	45.08 ± 2.94	45.22 ± 2.80	44.90 ± 2.96	44.04 ± 3.63
	Weight (g)	M/F	1.21 ± 0.24	1.38 ± 0.22	1.13 ± 0.23	1.17 ± 0.21	1.23 ± 0.25	1.17 ± 0.25	1.09 ± 0.29
Erythrocyte density	Fresh erythrocyte (cell/mm ³ x 10 ⁵)	M/F	$6.03\pm3.90~^{\circ}$	4.71 ± 3.13 bc	$2.72\pm0.53~^{ab}$	$3.25\pm0.72~^{ab}$	$3.63\pm1.36~^{bc}$	$2.17\pm0.96~^{a}$	$2.80\pm0.94~^{\rm ab}$
Erythrocyte	Erythrocyte necrosis (%)	M/F	$11.53\pm9.58^{\circ}$	$14.91\pm11.62~\mathrm{cd}$	1.01 ± 0.59 ^a	$6.20\pm8.39~^{\rm b}$	$20.64\pm8.13~^{\rm d}$	$20.10\pm8.85~^{\rm d}$	5.03 ± 5.22 ^b
mortality	Erythrocyte apoptosis (%)	M/F	$7.16\pm4.16~^{\rm d}$	$4.91\pm6.88~^{\rm bc}$	$0.45\pm0.67~^{\rm a}$	$2.17\pm5.11~^{ab}$	$4.51\pm4.51~\mathrm{cd}$	$9.45\pm4.87~^{\rm d}$	$2.23\pm3.12~\mathrm{bc}$
Chromosomal damage	DNA content variation (CV)	M/F	0.30 ± 0.59 ^a	$0.51\pm0.49~^{\rm abc}$	$0.86\pm0.88~^{bcd}$	$1.14\pm0.62~^{\rm d}$	$1.06\pm0.55~^{dc}$	$0.19\pm0.60~^{a}$	$0.44\pm0.66~^{\rm ab}$
Alkaline come	t DNA strand breaks (TI %)	M/F	$8.18\pm2.96~^{\rm ac}$	6.80 ± 2.38 ^a	$9.81\pm3.58~^{\rm abc}$	$11.87\pm4.52~^{\rm b}$	12.48 ± 5.73 ^b	$11.80\pm6.08~^{bc}$	$10.06\pm4.15~^{abc}$
assay	Hedgehogs (%)	M/F	NA	0.85 ± 0.95	1.11 ± 1.23	4.18 ± 4.58	1.50 ± 1.73	1.00 ± 1.75	0.89 ± 1.19
- د -	DNA strand breaks (TI %)	M/F	$15.72\pm5.93~^{ab}$	12.27 ± 3.72 a	$22.02\pm 6.62~^{\text{cde}}$	$20.93\pm6.95~\mathrm{cd}$	$25.72\pm6.37~\mathrm{de}$	35.23 ± 15.51 °	$19.47\pm6.59~\mathrm{bc}$
Fpg-modified	Hedgehogs (%)	M/F	NA	4.30 ± 4.05	2.52 ± 2.86	7.96 ± 13.16	5.09 ± 8.07	9.50 ± 19.65	4.56 ± 6.70
counce assay	Net Fpg-sensitive sites (TI %)	M/F	$7.64\pm6.79~^{ab}$	5.53 ± 3.66 ^a	$12.21\pm 5.56\ {\rm b}$	$9.07\pm5.98~^{ab}$	13.60 ± 5.03 ^b	$23.43\pm10.93~^\circ$	$9.47\pm7.59~^{ab}$
36 * Data M	VF indicates no significant impact of sex	t on any o	f parameters studied.						

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

est (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 obtaine	tandard length = 46.19 ± 3.02 mm, Weight = 1.21 ± 0.24 g and Fulton's condition = 1.22 ± 0.17 .	
	(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	(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 ndard length = 46.19 ± 3.02 mm, Weight = 1.21 ± 0.24 g and Fulton's condition = 1.22 ± 0.17 .

on Fulton's condition index T21	Mean ± Sd	$3 1.50 \pm 0.15 *$	$8 1.24 \pm 0.17 ***$	$6 1.27 \pm 0.13 **$	$8 1.32 \pm 0.13 *$	4 1.28 \pm 0.15	
Fulton's conditi index T0	Mean \pm Sd	1.41 ± 0.13	1.50 ± 0.18	1.40 ± 0.16	1.42 ± 0.18	1.34 ± 0.14	
Weight T21 (g)	Mean \pm Sd	1.38 ± 0.22	$1.13 \pm 0.23 **$	$1.18 \pm 0.21 *$	1.23 ± 0.26	1.17 ± 0.25	
Weight T0 (g)	Mean \pm Sd	1.30 ± 0.18	1.36 ± 0.27	1.32 ± 0.24	1.32 ± 0.29	1.15 ± 0.22	
Standard length T21 (mm)	Mean \pm Sd	45.09 ± 1.78	45.00 ± 2.88	45.08 ± 2.94	45.22 ± 2.80	44.90 ± 2.96	
Standard length T0 (mm)	Mean \pm Sd	45.04 ± 1.78	44.85 ± 2.49	45.58 ± 2.76	45.15 ± 2.96	44.00 ± 2.76	
		Manqueville	St Rémy du Nord	Artres	Biache-Saint-Vaast	Courrières	