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DNA damage in fish (*Anguilla anguilla*) exposed to a glyphosate-based herbicide – Elucidation of organ-specificity and the role of oxidative stress

S. Guilherme[a,*], I. Gaivão[b], M.A. Santos[a], M. Pacheco[a]

[a] Department of Biology and CESAM, University of Aveiro, 3810-193 Aveiro, Portugal
[b] CECAV and Department of Genetics and Biotechnology, Trás-os-Montes and Alto Douro University, Vila Real, Portugal

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ABSTRACT

Organophosphate herbicides are among the most dangerous agrochemicals for the aquatic environment. In this context, Roundup®, a glyphosate-based herbicide, has been widely detected in natural water bodies, representing a potential threat to non-target organisms, namely fish. Thus, the main goal of the present study was to evaluate the genotoxic potential of Roundup® in the teleost fish *Anguilla anguilla*, addressing the possible causative involvement of oxidative stress. Fish were exposed to environmentally realistic concentrations of this herbicide (58 and 116 $\mu g\,L^{-1}$) during one or three days. The standard procedure of the comet assay was applied to gill and liver cells in order to determine organ-specific genetic damage. Since liver is a central organ in xenobiotic metabolism, nucleoids of hepatic cells were also incubated with a lesion-specific repair enzyme (formamidopyrimidine DNA glycosylase – FPG), in order to recognise oxidised purines. Antioxidants were determined in both organs as indicators of pro-oxidant state. In general, both organs displayed an increase in DNA damage for the two Roundup® concentrations and exposure times, although liver showed to be less susceptible to the lower concentration. The enzyme-modified comet assay showed the occurrence of FPG-sensitive sites in liver only after a 3-day exposure to the higher Roundup® concentration. The antioxidant defences were in general unresponsive, despite a single increment of catalase activity in gills (116 $\mu g\,L^{-1}$, 3-day) and a decrease of superoxide dismutase activity in liver (58 $\mu g\,L^{-1}$, 3-day). Overall, the mechanisms involved in Roundup®-induced DNA strand-breaks showed to be similar in both organs. Nevertheless, it was demonstrated that the type of DNA damage varies with the concentration and exposure duration. Hence, after 1-day exposure, an increase on pro-oxidant state is not a necessary condition for the induction of DNA-damaging effects of Roundup®. By increasing the duration of exposure to three days, ROS-dependent processes gained preponderance as a mechanism of DNA-damage induction in the higher concentration.

1. Introduction

The increasing use of pesticides in contemporary agriculture is considered a major problem worldwide. Although the application of these agrochemicals is concentrated in terrestrial areas, they can reach the aquatic environment by drift, runoff, drainage and leaching [1], raising a number of environmental concerns especially in systems of shallow waters. Among pesticides, organophosphates constitute the predominant class [2]. In this context, the use of Roundup®, a glyphosate-based non-selective herbicide, has increased mainly due to the cultivation of genetically modified crops [3]. As a consequence of the extensive use of this commercial formulation, glyphosate has been widely detected in water bodies [4–7], increasing significantly the risks to non-target organisms, namely fish [8].

Although some studies have considered glyphosate to be only slightly toxic for aquatic animals [9,10] and with low potential to bio-accumulate [10], glyphosate-based formulations are generally more toxic than pure glyphosate [11,12], mainly due to the interference of surfactants [13]. Despite the existence of many studies concerning the deleterious effects of Roundup® on fish, only a few addressed its genotoxic potential. The available data showed genotoxicity of Roundup® to fish, expressed as cytogenetic and DNA-damaging effects [8,14,15]. Nevertheless, the concentrations tested in these studies were excessively high compared with the levels detected in natural water bodies. In addition, the mechanisms behind genetic damage and organ-specificities remain almost unexplored. Only recently, the association of Roundup® genotoxicity with oxidative stress was investigated for the first time in fish, following short-term exposure to environmentally realistic concentrations [16].

Elevated levels of reactive oxygen species (ROS) and/or depressed antioxidant defences may result in DNA oxidation and increased steady-state levels of unrepaired DNA, which is a

* Corresponding author.
E-mail address: sofia.g.guilherme@ua.pt (S. Guilherme).

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well-known process underlying genotoxicity, in particular in the context of environmental genotoxicants [17,18]. Since organophosphate pesticides are known as inducers of oxidative stress [19], the hypothesis that DNA damage induced by Roundup® may also have an oxidative cause should be considered. This association has already been demonstrated in humans for organophosphate pesticides [20]. In relation to fish, the only available study demonstrated that DNA and chromosomal damage induced by Roundup® in blood cells was not paralleled by an increased pro-oxidant state, as evaluated by antioxidant responses [16]. This study also recommended the assessment of oxidation of DNA bases (for instance, by applying the comet assay with an extra digestion step of the nucleoids, with enzymes that specifically recognise oxidised bases) as a more straightforward strategy to obtain the required mechanistic knowledge.

Genotoxic studies in fish are frequently performed in erythrocytes, due to the ease of sampling and their adaptability to the most common methodologies [21,22]. However, according to Sharma et al. [23], other cell types should be used for monitoring genotoxic effects, thereby exploiting tissue-specific responses and acquiring a better perspective about the overall condition of the organisms. When waterborne contamination is considered, gills are the first target organ due to the large surface area in direct and continuous contact with the external medium, and its involvement in uptake [24,25]. Additionally, the liver is also of great interest for health assessment of individual fish in view of its multi-functionality and its primary role in the metabolism of xenobiotics, which is essential for activation and inactivation/detoxification of contaminants absorbed via different routes [26]. Moreover, exposure of fish to Roundup® induced histological injuries in both gills and liver [27], despite the fact that antioxidant alterations were only demonstrated in liver [28,29].

Considering that genotoxicity stands for a strongly adverse impact of chemicals on wild organisms and in view of the knowledge gaps previously recognised, the main goal of the present study was to evaluate the genotoxic potential of Roundup® to gill and liver cells of fish (*Anguilla anguilla*), following short-term exposure to environmentally realistic concentrations (58 and 116 $\mu g\,L^{-1}$), addressing the possible causative involvement of oxidative stress. The standard procedure of the comet assay was applied to gill and liver cells in order to reflect organ-specific genetic damage. Additionally, and considering the peculiarities of liver in fish physiology, the comet assay with an extra step where nucleoids are incubated with a DNA lesion-specific repair enzyme (formamidopyrimidine DNA glycosylase – FPG) was applied to hepatic cells in order to specifically target oxidised DNA bases. Superoxide dismutase (SOD), catalase (CAT), glutathione-*S*-transferase (GST), glutathione peroxidase (GPx) and glutathione reductase (GR) activities, as well as total glutathione (GSHt) content, were determined in both organs as indicators of pro-oxidant state.

2. Material and methods

2.1. Chemicals

A commercial formulation of glyphosate (Roundup® Ultra, distributed by Bayer CropScience, Portugal), containing isopropylammonium salt of glyphosate at 485 $g\,L^{-1}$ as the active ingredient (equivalent to 360 $g\,L^{-1}$ or 30.8% of glyphosate) and polyethoxylene amine (16%) as surfactant, was used. Formamidopyrimidine DNA glycosylase was purchased from Andrew Collins, University of Oslo, Norway. All the other chemicals required to perform the comet assay and to quantify antioxidants were obtained from Sigma–Aldrich Chemical Company (Spain).

2.2. Test animals and experimental design

European eel (*A. anguilla* L.) specimens with an average length of 25 ± 3 cm and weight of 32 ± 5 g (yellow eel stage) were captured from an unpolluted area of the Aveiro Lagoon – Murtosa, Portugal. Eels were acclimated to laboratory for 12 days and kept in 80-L aquaria under a natural photoperiod, in aerated, filtered, de-chlorinated and recirculating tap water, with the following physico-chemical conditions: salinity 0, temperature $20 \pm 1\,^{\circ}C$, pH 7.3 ± 0.2, ammonia <0.1 $mg\,L^{-1}$, nitrite 0.06 ± 0.03 $mg\,L^{-1}$, nitrate 25 ± 6.0 $mg\,L^{-1}$, dissolved oxygen 8.1 ± 0.5 $mg\,L^{-1}$. During this period, fish were fed every other day with fish roe.

The experiment was carried out in 20-L aquaria, in a static mode. Physical-chemical characteristics of the water during the experiment were daily monitored and fell in the intervals described above for the acclimation period. Fish were not fed one day before the experiment was started, or during the experimental period. Thirty-six eels were divided over six aquaria (six fish per dose per duration group; $n = 6$) and exposed to 58 $\mu g\,L^{-1}$ (two aquaria) and 116 $\mu g\,L^{-1}$ (two aquaria) of Roundup®, equivalent to 18 and 36 $\mu g\,L^{-1}$ of glyphosate, respectively. Another two aquaria were kept with clean water as negative control groups. For each pesticide concentration, 1- and 3-day exposures were tested, corresponding to the two different aquaria mentioned above. No mortality was observed during the whole experiment. After each exposure period, fish were sacrificed by cervical transection and bled. Liver and gills were collected and washed in ice-cold phosphate-buffered saline (PBS). A tissue portion of each organ was immediately processed for the comet assay and the remaining tissue was stored in micro-tubes, frozen in liquid nitrogen and kept at $-80\,^{\circ}C$ until further procedures for analysis of antioxidants.

2.3. Evaluation of genetic damage

2.3.1. Comet assay

Liver and gill cell suspensions were obtained by mincing briefly a part of the tissue with a pair of fine scissors in 1 mL of PBS and by pipetting up-and-down the finely minced tissue pieces [30]. The conventional alkaline version of the comet assay was performed according to the method of Collins [18] with slight modifications. Two gel replicates, containing each approximately 2×10^4 cells (cell suspension in PBS) in 70 μL of 1% low melting-point agarose in PBS, were placed on a glass microscope slide, pre-coated with 1% normal melting-point agarose. The gels were covered with glass coverslips and left for ±5 min at 4 °C to let the agarose solidify, and then immersed in a lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 1% Triton X-100, pH 10) at 4 °C, for one hour. Then, slides were gently placed in the electrophoresis tank, immersed in electrophoresis solution (±20 min, 0.3 M NaOH, 1 mM EDTA, pH > 13) for alkaline treatment. Electrophoresis was performed at a fixed voltage of 25 V and a current of 300 mA, which results in 0.7 $V\,cm^{-1}$ (achieved by adjusting the buffer volume in the electrophoresis tank). The slides were stained with ethidium bromide (20 $\mu g\,L^{-1}$).

For the liver, an additional set of slides was prepared to apply the comet assay with an extra step of digesting the nucleoids with FPG. This lesion-specific endonuclease converts oxidised purines, including the major purine oxidation product 8-oxoguanine and other altered purines (ring-opened purines or formamido-pyrimidines) into DNA single-strand breaks [17]. Thus, after lysis of agarose-embedded cells, slides were washed three times with enzyme buffer (0.1 M KCl, 0.5 mM EDTA, 40 mM HEPES, 0.2 $mg\,mL^{-1}$ bovine serum albumin, pH 8) at 4 °C. Then, 50 μL of FPG in buffer was applied in the centre of each gel, along with a coverslip, prior to incubation at 37 °C for 45 min in a humidified atmosphere. Another set of slides was submitted to the same treatment, although incubated only with buffer. Subsequent steps – alkaline treatment, electrophoresis and staining – were as described above.

One slide with two gels each, and 100 nucleoids per gel, were observed for each fish and organ, with a Leica DMLS fluorescence microscope (400× magnification). The DNA damage was quantified by visual classification of nucleoids into five comet classes, according to the tail intensity and length, from 0 (no tail) to 4 (almost all DNA in tail). The total score expressed as a genetic damage indicator (GDI) was calculated multiplying the mean percentage of nucleoids in each class by the corresponding factor, according to the formula:

$$\begin{aligned}\text{GDI} = {} & [(\%\,\text{nucleoids class 0}) \times 0] + [(\%\,\text{nucleoids class 1}) \times 1] \\ & + [(\%\,\text{nucleoids class 2}) \times 2] + [(\%\,\text{nucleoids class 3}) \times 3] \\ & + [(\%\,\text{nucleoids class 4}) \times 4]\end{aligned}$$

GDI results were expressed as arbitrary units on a scale of 0–400 per 100 scored nucleoids (as average value for the two gels observed per fish). When the comet assay was performed with the additional FPG step (for liver), GDI values were calculated in the same way but the parameter designated GDI_{FPG}. Besides the GDI, the frequency of nucleoids observed in each comet class was also expressed, as recommended by Azqueta et al. [17]. In order to improve the expression of the extent of DNA damage, the sub-total frequency of nucleoids with medium (class 2), high (class 3) and complete (class 4) damaged DNA was also calculated [8,31].

As positive controls, both gill and liver cells were treated with 50 μM hydrogen peroxide (Sigma–Aldrich, Spain) for 5 min, according to Collins et al. [32], and the respective GDI values were scored.

2.4. Antioxidant system analyses

2.4.1. Tissue preparation and fractionation

Both organs (gills and liver) were homogenized in a 1:10 ratio (tissue volume: buffer volume) with a Potter-Elvehjem homogenizer, in chilled phosphate buffer

(0.2 M, pH 7.4). The homogenate was then divided into two aliquots: for GSHt quantification and for post-mitochondrial supernatant (PMS) preparation, to be used in the enzymatic determinations. The PMS fraction was obtained by centrifugation in a refrigerated centrifuge (Eppendorf 5415R) at 13,400 × *g* for 20 min at 4 °C. Aliquots of PMS were stored in micro-tubes at −80 °C until analysis.

2.4.2. Measurement of antioxidant responses

Superoxide dismutase was assayed (at 25 °C) with a Ransod kit (Randox Laboratories Ltd., UK). The method employs xanthine and xanthine oxidase to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. SOD activity is then measured by the degree of inhibition of this reaction. One unit of SOD is the amount that causes a 50% inhibition of the rate of reduction of INT, under the conditions of the assay. Results were expressed as SOD units mg^{-1} protein.

Catalase activity was assayed (at 25 °C) by the method of Claiborne [33] as described by Giri et al. [34]. Briefly, the assay mixture consisted of 1.95 mL phosphate buffer (0.05 mol L^{-1}, pH 7.0), 1 mL hydrogen peroxide (0.019 mol L^{-1}) and 0.05 mL of sample in a final volume of 3 mL. Change in absorbance was recorded spectrophotometrically at 240 nm and CAT activity was calculated in terms of μmol H_2O_2 consumed min^{-1} mg^{-1} protein ($\varepsilon = 43.5\ M^{-1}\ cm^{-1}$).

Glutathione-*S*-transferase activity was determined with CDNB (1-chloro-2,4-dinitrobenzene) as a substrate, according to the method of Habig et al. [35]. The assay was carried out at 25 °C in a quartz cuvette with a 2 mL mixture of 0.2 M phosphate buffer (pH 7.4), 0.2 mM CDNB and 0.2 mM reduced glutathione (GSH). The reaction was initiated by addition of 0.01 mL of sample, and the increase in absorbance was recorded spectrophotometrically (Jasco UV/VIS, V-530) at 340 nm, for 3 min. The enzyme activity was calculated as nmol CDNB conjugate formed min^{-1} mg^{-1} protein ($\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$).

Glutathione peroxidase activity was determined (at 25 °C) according to the method of Mohandas et al. [36], with some modifications. The assay mixture consisted of 0.72 mL phosphate buffer (0.05 M, pH 7.0), 0.05 mL EDTA (1 mM), 0.05 mL sodium azide (1 mM), 0.025 mL GR (1 IU mL^{-1}), 0.05 mL GSH (4 mM), 0.05 mL NADPH (0.8 mM), 0.005 mL H_2O_2 (1.0 mM) and 0.05 mL of sample in a total volume of 1 mL. NADPH oxidation was recorded spectrophotometrically at 340 nm, and GPx activity was calculated in terms of nmol NADPH oxidised min^{-1} mg^{-1} protein ($\varepsilon = 6.22 \times 10^3\ M^{-1}\ cm^{-1}$).

Glutathione reductase activity was assayed (at 25 °C) by the method of Cribb et al. [37], with some modifications. The assay determines indirectly the GR activity by measuring the NADPH disappearance associated with reduction of oxidised glutathione (GSSG) catalysed by GR. Briefly, the assay mixture contained 0.025 mL of PMS fraction and 0.975 mL of NADPH (0.2 mM), GSSG (1 mM) and diethylenetriaminepentaacetic acid (DTPA) (0.5 mM). Change in absorbance at 340 nm was registered spectrophotometrically (Jasco UV/VIS, V-530) during 3 min and GR activity calculated as nmol of NADPH oxidised min^{-1} mg^{-1} protein ($\varepsilon = 6.22 \times 10^3\ M^{-1}\ cm^{-1}$).

For GSHt quantification, protein in the tissue lysate was precipitated with trichloroacetic acid (TCA 12%) for 1 h and then centrifuged at 13,400 × *g* for 20 min at 4 °C. The resulting supernatant was collected and stored at −80 °C. GSHt was determined (in deproteinated PMS, at 25 °C) by adopting the enzymatic recycling method using GR excess, whereby the sulfhydryl group of GSH reacts with 5,5-dithiobis-tetranitrobenzoic acid and produces a yellow 5-thio-2-nitrobenzoic acid (TNB). The rate of TNB production is directly proportional to the concentration of glutathione in the sample [38,39]. Formation of TNB was measured by spectrophotometry (Jasco UV/VIS, V-530) at 412 nm. It should be noted that GSSG in this system is converted to GSH by GR, which, consequently, measures total glutathione (GSHt) content. The results were expressed as nmol TNB formed min^{-1} mg^{-1} protein ($\varepsilon = 14.1\ mM^{-1}\ cm^{-1}$).

Total protein content was determined according to the Biuret method [40], with bovine serum albumin (Merck) as a standard.

2.5. Statistical analysis

SigmaStat software (SPSS Inc.) was used for statistical analyses. All data were first tested for normality and homogeneity of variance to meet statistical demands. One-way ANOVA analysis was used to compare the different treatments within the same exposure duration, and to compare the same treatment in different exposure durations. The Tukey test was applied for post hoc comparison. Whenever the assumptions for parametric statistics failed, a non-parametric corresponding test (Kruskall Wallis) was performed, followed by a non-parametric all pairwise multiple-comparison procedure (Dunn's test) [41].

3. Results

3.1. DNA damage

3.1.1. Gills

Gills of fish exposed to both concentrations of Roundup® (58 and 116 μg L^{-1}) demonstrated an increase in GDI values, after 1- and 3-day exposures, when compared to respective controls (Fig. 1). Concerning 1-day exposure, gills GDI presented a 1.6- and 1.7-fold increase, respectively for 58 and 116 μg L^{-1} Roundup® concentrations, when compared with control. After three days, GDI values were 1.4 and 1.8 times increased, respectively for the 58 and 116 μg L^{-1} dose groups. Moreover, the 116 μg L^{-1} group displayed a significant GDI increase, when compared with the 58 μg L^{-1} group. Overall, the GDI showed to be concentration-dependent, whereas only one time-related alteration was noticeable, i.e. a decrease from the 1-day to the 3-day exposure for the concentration of 58 μg L^{-1} of Roundup®. The positive control (cells treated with H_2O_2) displayed an average GDI of 291.7 (±8.28) arbitrary units, showing to be significantly higher than the negative control and both Roundup treatments.

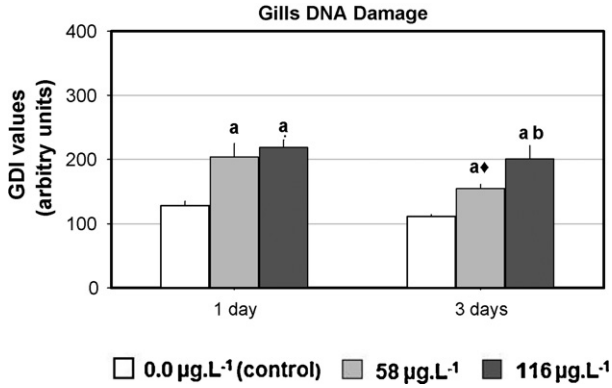

Fig. 1. Mean values of genetic damage indicator (GDI), expressed as arbitrary units, measured by comet assay in gills of *A. anguilla* exposed to 58 and 116 μg L^{-1} Roundup®, during 1 and 3 days. Statistically significant differences ($p < 0.05$) are: (a) vs. control and (b) vs. 58 μg L^{-1} (within the same exposure duration); (♦) vs. 1-day exposure (for the same exposure condition). Bars represent the standard error.

The results in terms of individual DNA-damage classes are presented in Table 1. After the first day of exposure, gills of fish exposed to 58 μg L^{-1} of Roundup® showed significant increases in classes 2 and 4 when compared with control, while in the 116 μg L^{-1} group significant increases were detected in classes 2, 3 and 4. The sub-total of damaged nucleoids (sum of damage classes 2, 3 and 4) showed increments of 3 and 3.5 times, respectively for the 58 and 116 μg L^{-1} groups, when compared with control, highlighting an influence of the Roundup® concentration in the magnitude of damage. Following the 3-day exposure to 58 μg L^{-1} of Roundup®, only class 2 showed a significant increase in comparison with the control. After 3-day exposure to 116 μg L^{-1}, classes 2 and 3 showed significant increases. Significant time-related differences were observed in classes 1 (increase) and 4 (decrease). The frequency of damaged nucleoids (sub-total 2 + 3 + 4) was significantly elevated in both treatment groups (4.7- and 6.6-fold, respectively, for 58 and 116 μg L^{-1}), although it seems to decrease in comparison with the corresponding levels after 1-day exposure (significantly lower for the 58 μg L^{-1} group). Overall, and considering both Roundup® concentrations, class 2 was the most prevalent following 1-day exposure, whereas after 3-day exposure the most prevalent was class 1.

3.1.2. Liver

After 1-day exposure, liver of fish treated with the two Roundup® concentrations (Fig. 2A) displayed significantly higher GDI values, in relation to the control. The increments were around 1.5 and 1.6 times, respectively for 58 and 116 μg L^{-1}. With respect to the 3-day exposure, only the higher concentration showed a significant GDI increase (1.6 times), when compared with the control. This group also showed a significant increase (1.7-fold) in relation to the 58 μg L^{-1} group. Considering the GDI results as a whole, a

Table 1
Mean frequencies (%) of each DNA damage class and sub-total of damaged nucleoids (±standard error), measured by comet assay, in gill cells of *A. anguilla* exposed to 58 and 116 μg L^{-1} Roundup®, during 1 and 3 days. Statistically significant differences ($p < 0.05$) are: (a) vs. control (within the same exposure duration); (♦) vs. 1-day exposure (for the same exposure condition).

Exposure time	Roundup® concentration (μg L^{-1})	Gills DNA Damage Classes 0	1	2	3	4	Sub-total (2+3+4)
1 day	0 (control)	0.00 ± 0.00	76.80 ± 4.52	18.90 ± 3.39	3.50 ± 0.94	0.80 ± 0.49	23.20 ± 4.52
	58	0.00 ± 1.40	29.60 ± 6.93[a]	45.80 ± 3.01[a]	15.70 ± 4.07	8.90 ± 1.06[a]	70.40 ± 10.31[a]
	116	0.00 ± 0.00	18.00 ± 6.69[a]	50.80 ± 4.73[a]	21.60 ± 4.78[a]	9.60 ± 2.37[a]	82.00 ± 6.69[a]
3 days	0 (control)	0.10 ± 0.10	90.20 ± 2.28[♦]	7.80 ± 2.32[♦]	1.90 ± 0.48	0.00 ± 0.00	9.70 ± 2.31[♦]
	58	0.00 ± 0.00	54.30 ± 2.58[a♦]	37.80 ± 2.31[a]	6.50 ± 1.90	1.40 ± 0.94[♦]	45.70 ± 2.58[a♦]
	116	0.00 ± 0.00	35.50 ± 8.30[a]	34.40 ± 5.43[a]	23.40 ± 7.81[a]	6.70 ± 3.12	64.50 ± 8.30[a]

concentration-dependence was not clear. Differently, time-related variations included a significant decrease in the 58 μg L^{-1} group and an increase in the 116 μg L^{-1} group. The positive control displayed an average GDI of 283.0 (±11.80) arbitrary units, i.e.

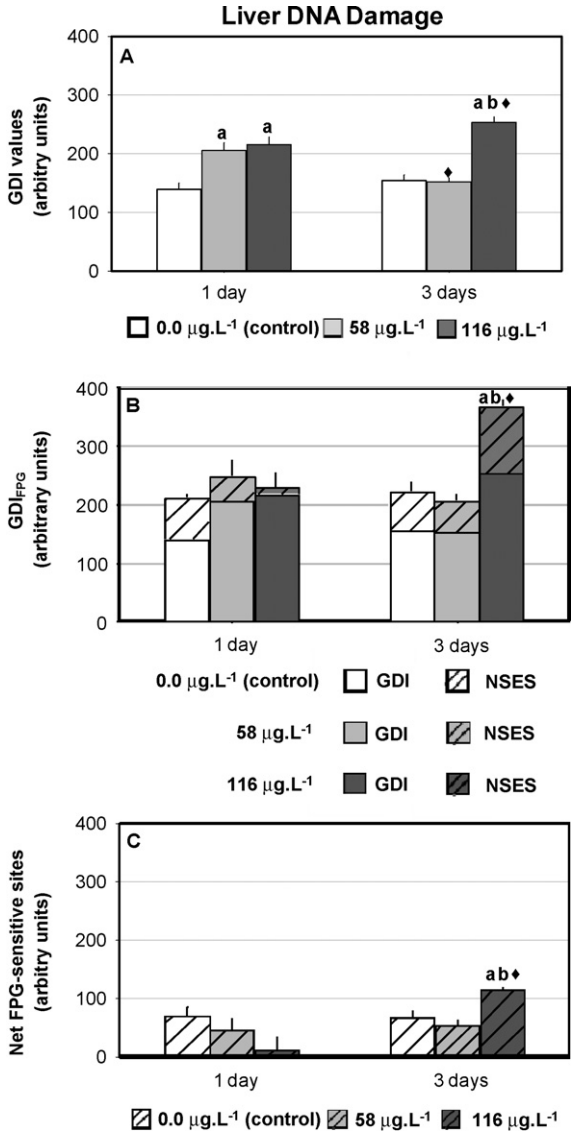

Fig. 2. Mean values of DNA damage, expressed in arbitrary units, measured by comet assay in liver of *A. anguilla* exposed to 58 and 116 μg L^{-1} Roundup®, during 1 and 3 days. (A) Genetic damage indicator (GDI) after standard (alkaline) comet assay. Values after enzyme-modified comet assay, as a measure of bases oxidation, showing overall (GDI_{FPG}) and partial scores (B), as well as additional DNA breaks corresponding to net FPG-sensitive sites (calculated by the difference between GDI_{FPG} and GDI values) (C). Statistically significant differences ($p < 0.05$) are: (a) vs. control and (b) vs. 58 μg L^{-1} (within the same exposure duration); (♦) vs. 1-day exposure (for the same exposure condition). Bars represent the standard error.

significantly higher than the negative control and both Roundup treatments.

Concerning the frequency of individual classes of damage (Table 2), after 1-day exposure to 58 μg L^{-1} none of the classes showed a significant change. However, a significant increase (approximately 1.7-fold) was found in the sub-total of damaged nucleoids for this group compared with the control. On the other hand, the 116 μg L^{-1} group exhibited significant increases (compared with the control) either in classes 3 and 4, or in the sub-total of damaged nucleoids (about 1.6-fold). Both Roundup® concentrations caused a significant decrease in the frequency of class 1, compared with the control. The results of 3-day exposure revealed significant changes only for the higher herbicide concentration. Thus, the 116 μg L^{-1} group showed significantly higher frequencies of classes 3 and 4, and of the sub-total of damaged nucleoids, compared with the control and the 58 μg L^{-1} groups.

Some differences were also found comparing both exposure times. A time-related decrease was observed for class 2 and for the sub-total of damaged nucleoids in the 58 μg L^{-1} group as well as for class 1 in the 116 μg L^{-1} group, whereas an opposite temporal variation was observed for class 1 in the 58 μg L^{-1} group and for the sub-total of damaged nucleoids in the 116 μg L^{-1} group.

When the digestion with FPG enzyme was incorporated in the assay, significant differences were only found after the 3-day exposure for the 116 μg L^{-1} group (Fig. 2B and C). Taking into account the overall score (Fig. 2B), this group showed significant increases (1.7- and 1.8-fold) compared with the controls and the lower concentration group, respectively. Considering the net FPG-sensitive sites (Fig. 2C), the higher concentration group (116 μg L^{-1}) showed increases of 1.7- and 2.1-fold when compared with control and with the lower concentration, respectively. Moreover, the 116 μg L^{-1} group showed significant increases from 1- to 3-day exposure for both overall score and net FPG-sensitive sites, being the increase particularly relevant in the latter parameter (10-fold).

3.2. Antioxidant responses

3.2.1. Gills

Concerning the antioxidant responses measured in both Roundup®-treated groups (Fig. 3), a significant increase was only found for CAT activity in the 116 μg L^{-1} group after 3-day exposure, compared either with the control or with the 58 μg L^{-1} group (Fig. 3B). Regarding the comparison between the 1- and 3-day exposures, a significant time-related decrease in GPx activity is noted in both treated groups (Fig. 3E), as well as in GSHt content in the 116 μg L^{-1} group (Fig. 3F).

3.2.2. Liver

With the exception of a significant decrease in SOD activity in the liver of the 58 μg L^{-1} group after the 3-day exposure (Fig. 4A), no alterations were observed in antioxidant responses (Fig. 4).

Table 2
Mean frequencies (%) of each DNA damage class and sub-total of damaged nucleoids (± standard error), measured by comet assay, in liver cells of *A. anguilla* exposed to 58 and 116 μg L^{-1} Roundup®, during 1 and 3 days. Statistically significant differences ($p < 0.05$) are: (a) vs. control and (b) vs. 58 μg L^{-1} (within the same exposure duration); (♦) vs. 1-day exposure (for the same exposure condition).

Exposure time	Roundup® concentration (μg L^{-1})	Liver DNA damage classes 0	1	2	3	4	Sub-total (2+3+4)
1 day	0.00 (control)	0.00 ± 0.00	55.60 ± 7.85	38.40 ± 5.71	4.80 ± 2.02	1.20 ± 1.08	44.40 ± 7.85
	58	0.00 ± 0.00	24.40 ± 5.70[a]	52.00 ± 2.81	17.50 ± 6.21	6.10 ± 1.97	75.60 ± 5.70[a]
	116	0.40 ± 0.00	29.50 ± 4.42[a]	35.90 ± 3.08[b]	22.40 ± 3.26[a]	11.80 ± 4.07[a]	70.10 ± 4.55[a]
3 days	0.00 (control)	0.00 ± 0.00	57.00 ± 5.94	33.20 ± 3.41	8.20 ± 1.91	1.60 ± 0.97	43.00 ± 5.94
	58	0.00 ± 0.00	57.10 ± 4.35♦	35.20 ± 3.85♦	6.30 ± 1.51	1.40 ± 1.28	42.90 ± 4.35♦
	116	0.00 ± 0.00	7.50 ± 2.20[ab]♦	47.20 ± 4.19	29.60 ± 2.46[ab]	15.70 ± 3.82[ab]	92.50 ± 2.20[ab]♦

4. Discussion

The intentional application of Roundup® or other glyphosate-based formulations to control emergent and floating aquatic vegetation can result in greater localized concentrations in aquatic systems than those from runoff from terrestrial uses [3]. Giesy et al. [3] developed a model to estimate the worst-case exposure conditions. Taking into account this theoretical model, values in the ranges 0.27–0.41 and 0.34–0.68 mg L^{-1} of Roundup® were considered the maximum concentrations likely to be found in surface waters following terrestrial uses or direct applications, respectively. In general, these estimates have proven to be correct, since concentrations of glyphosate were detected in the range 75–90 μg L^{-1} in the Orge watershed (France) [42] and higher levels (0.5–1.0 mg L^{-1}) than those predicted were sporadically found following direct application to water [10]. Extreme

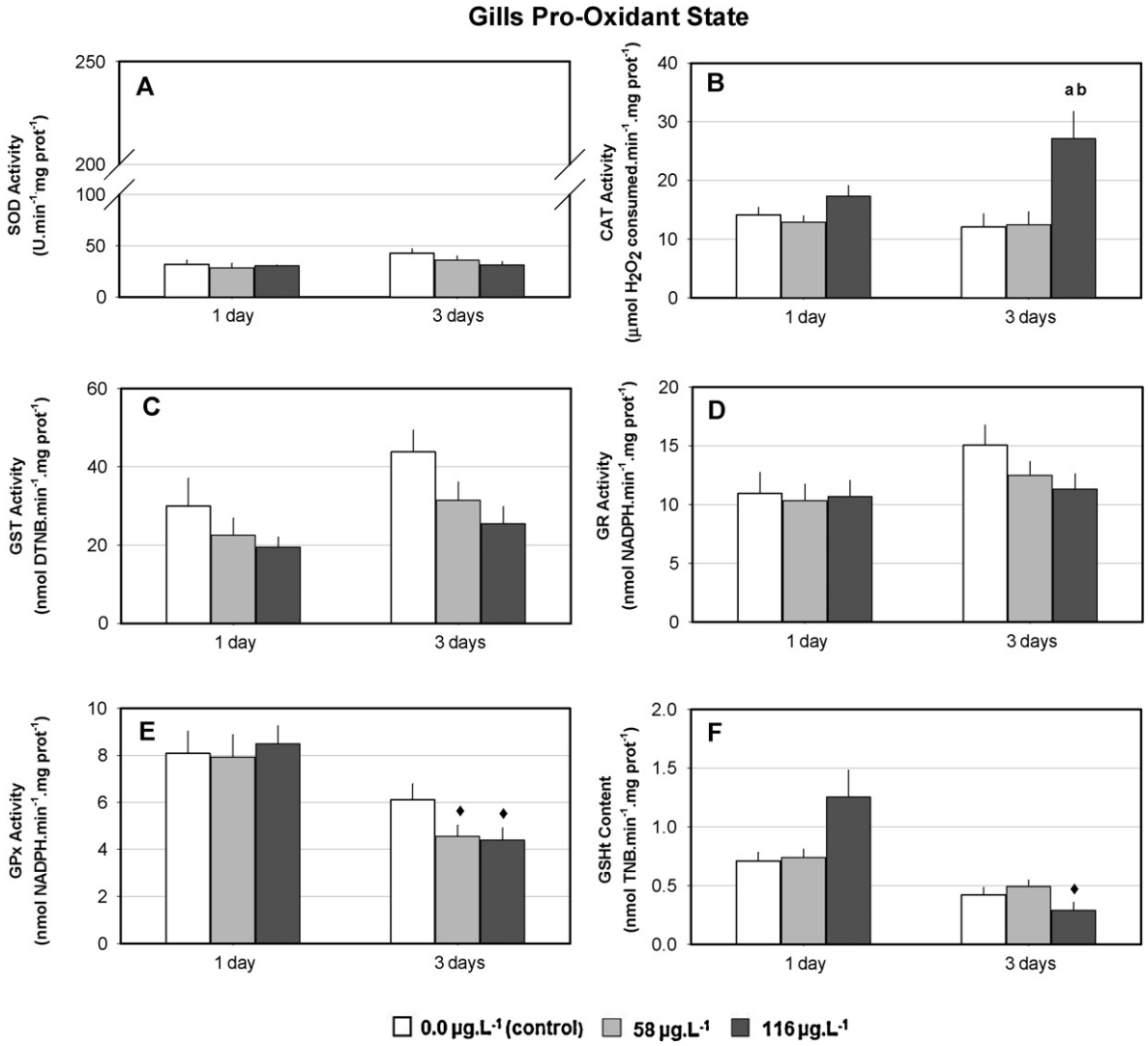

Fig. 3. Mean superoxide dismutase (SOD) (A), catalase (CAT) (B), glutathione-S-transferase (GST) (C), glutathione reductase (GR) (D) and glutathione peroxidase (GPx) (E) activities, as well as total glutathione (GSHt) content (F) in gills of *A. anguilla* exposed to 58 and 116 μg L^{-1} Roundup®, during 1 and 3 days. Statistically significant differences ($p < 0.05$) are: (a) vs. control and (b) vs. 58 μg L^{-1} (within the same exposure duration); (♦) vs. 1-day exposure (for the same exposure condition). Bars represent the standard error.

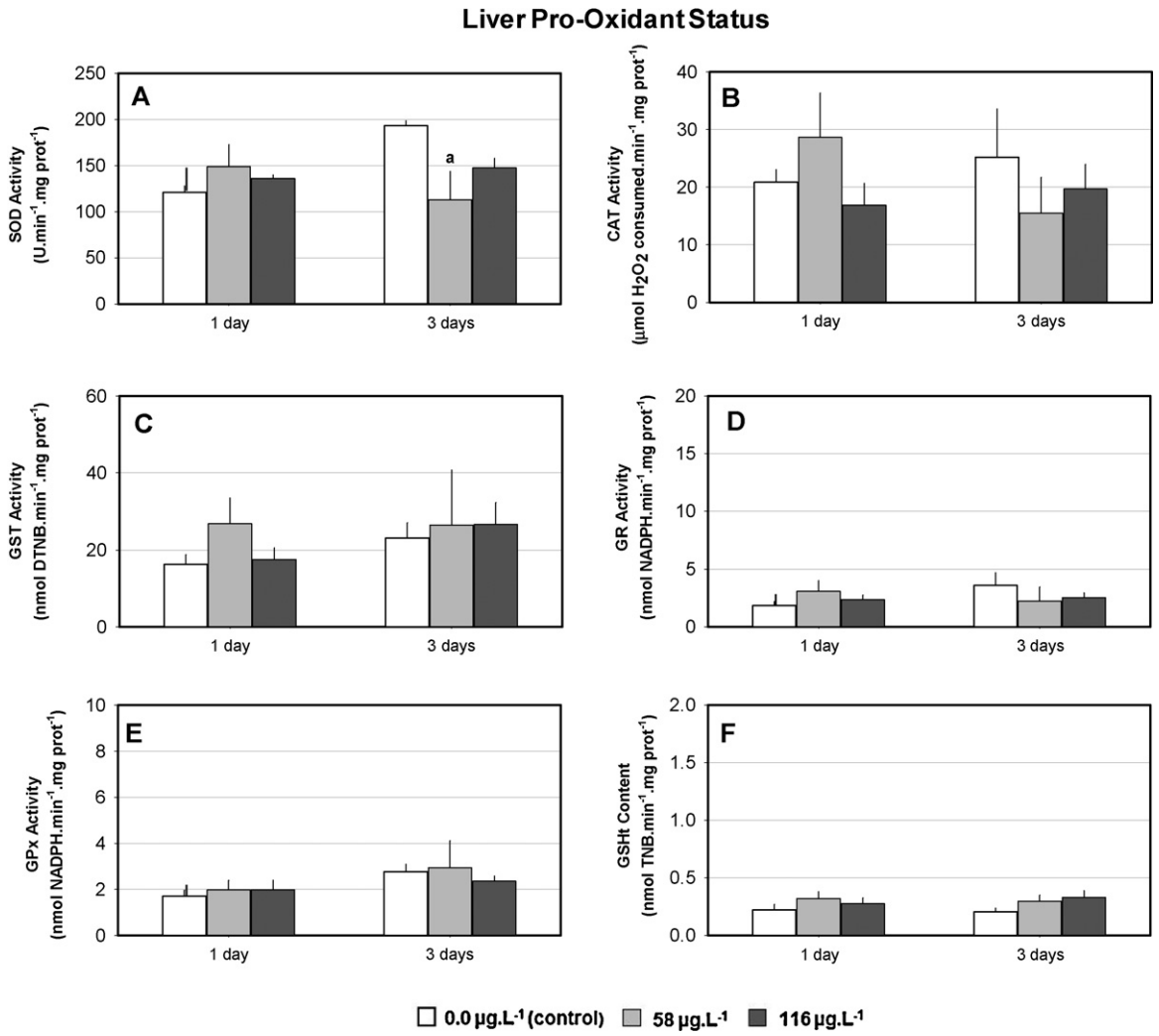

Fig. 4. Mean superoxide dismutase (SOD) (A), catalase (CAT) (B), glutathione-S-transferase (GST) (C), glutathione reductase (GR) (D) and glutathione peroxidase (GPx) (E) activities, as well as total glutathione (GSHt) content (F) in liver of *A. anguilla* exposed to 58 and 116 µg L^{-1} Roundup®, during 1 and 3 days. Statistically significant differences ($p < 0.05$) are: (a) vs. control. Bars represent the standard error.

values of glyphosate were found near agricultural areas in Brazil, corresponding to the range 0.36–2.16 mg L^{-1} of a commercial formulation of Roundup® (360 g L^{-1} glyphosate) [43].

The concentrations tested in the current research (corresponding to 18 and 36 µg L^{-1} of glyphosate) are realistic and are in contrast with other studies where Roundup® concentrations were one order of magnitude higher [8,14,25,28,29]. In addition, the present study represents an important progress compared with the few previous fish studies on Roundup® genotoxicity by its mechanistic approach and by the exploration of organ-specific susceptibilities. To the authors' knowledge, only one report is available on gills in this context [14] and no studies were yet performed on liver. Furthermore, the use of the comet assay in combination with a specific DNA-repair enzyme, FPG, is a novel approach, since this tool, applied for the first time to fish in 2003 [44], has never been used before to assess pesticide-induced DNA damage.

4.1. DNA damage and pro-oxidant state in gills

GDI results clearly indicated the potential of Roundup® to induce DNA strand-breaks in branchial cells at both test concentrations (58 and 116 µg L^{-1}) and exposure times (1 and 3 days). Overall, a concentration-dependence was observed mainly after a 3-day exposure. A time-related attenuation of the effect was perceptible only for the lower herbicide concentration (showing lower GDI values at day 3 in comparison with day 1, although still higher than the respective control), revealing a concentration-related pattern. This temporal variation can be explained by the reduced levels of the pesticide (or their metabolites) in gill tissue, combined with the intervention of DNA-repair system and/or cell turnover. In this direction, it was demonstrated that epithelium of the gills is regularly subject to exfoliation and erosion, which is counteracted by an intense cell-division rate [45].

Examining the individual damage classes, it was possible to identify in treated fish, invariably, decreases in class 1 (undamaged nucleoids) frequency, whenever damaged nucleoids classes (2, 3 and 4) predominantly presented increased frequencies. It is important to highlight the damaged nucleoids (sub-total 2 + 3 + 4) frequency, since it showed higher increments in treated groups (maximum 6.6 times above the control) when compared with the corresponding increments obtained for GDI values (maximum 1.7-fold). Rather than GDI, the damaged nucleoids frequency appears to have a higher capacity to discriminate between treated and untreated fish. Thus, the analysis of individual DNA-damage classes seems to improve the information concerning the magnitude of damage, making clearer concentration- and time-related response profiles.

The main outcome of current comet assay regarding *A. anguilla* gills is in agreement with data reported by Cavalcante et al. [14] in gills of the neotropical fish *Prochilodus lineatus*, where the potential of Roundup® to affect DNA integrity was also demonstrated after 6 and 24 h exposure to 10 mg L^{-1}.

Only recently the potential of Roundup® (or glyphosate) to induce oxidative stress responses in fish has been addressed [28,29,46,47]. The available studies provided inconclusive and divergent information, due to the variety of species and concentration ranges adopted, as well as the target organs analysed. Although Roundup® has been shown as an oxidative stress agent on different fish organs/tissues [28,29,47], its impact specifically on the pro-oxidant state of gills has never been addressed. On the other hand, it is documented that fish gills can be more vulnerable towards oxidative damage than other organs (e.g. liver) and may respond earlier to a pollutant-induced pro-oxidant challenge [48,49]. Therefore, DNA oxidation was hypothesised as a potential damage type induced by Roundup® in branchial cells. However, present data concerning 1-day exposure revealed that DNA strand-break induction was not accompanied by an increased pro-oxidant state, suggesting that DNA was not oxidatively damaged under these conditions. Differently, after a 3-day exposure, the higher concentration (116 μg L^{-1}) induced a CAT activity increase, indicating an overproduction of H_2O_2, the main cell precursor of the hydroxyl radical ($OH^{\bullet}$) which is considered to be the most toxic ROS. Hence, under these circumstances, DNA oxidation may play a role in the genotoxic effects of Roundup® as demonstrated in *A. anguilla* gills.

4.2. Liver DNA damage and underlying mechanisms

Following 1-day exposure, GDI results demonstrated that Roundup® affects DNA integrity of hepatic cells at both exposure concentrations, not revealing a concentration dependency. The exposure time extension revealed a different pattern, since at day 3, the GDI value for the lower concentration reversed to the control level, whereas the higher concentration exhibited a time-related GDI increase. As stated for gills, the analysis of individual DNA-damage classes reinforced the outcome obtained with GDI.

The clarification of the involvement of oxidative stress in the damaging effect of Roundup® on liver DNA was attempted by combining the analysis of antioxidant responses and the identification of additional DNA breaks corresponding to FPG-sensitive sites. The antioxidant system did not indicate an increased pro-oxidant state in liver, as both enzymatic and non-enzymatic antioxidants remained unchanged under all the exposure conditions. This finding agrees with a previous study performed by Mañas et al. [50] who observed that intra-peritoneal administration of glyphosate in mice caused genotoxicity in the liver, despite the absence of induction of antioxidant defences. In accordance, after 1-day exposure, no DNA oxidation was reflected in the results concerning overall GDI_{FPG} scores or net FPG-sensitive sites. Furthermore, the lowest value for net FPG-sensitive sites was measured in the 116 μg L^{-1} group after 1-day exposure, highlighting that under short exposures the base oxidation is not a relevant mechanism of damage. In contrast, following the 3-day exposure, oxidised purines were found to be elevated in the 116 μg L^{-1} group, as is clear from the significant increase of GDI_{FPG} and net FPG-sensitive sites observed in comparison with the control and 58 μg L^{-1} groups. Surprisingly, it should be noted that the Roundup-induced DNA oxidative damage observed in the enzyme-modified comet assay was not accompanied by activation of the antioxidant system. Thus, as previously stated [51], the oxidative damage cannot be predicted only on the basis of antioxidant variations. This association can be particularly compromised when the consumption of low molecular weight antioxidants is counterbalanced by *de novo* synthesis and/or when inhibitory actions impair the activity of enzymatic antioxidants. Taking into account the present results, the occurrence of this effect cannot be excluded, i.e. in view of the decrease in SOD activity detected after the 3-day exposure to 58 μg L^{-1}. Giving support to this observation, Lushchak et al. [29] found that exposure to Roundup® (2.5–20 mg L^{-1}) suppressed SOD activity in the liver of *Carassius auratus*, which was explained by a ROS-induced inactivation. It is also important to note that the currently observed SOD inhibition occurred for the only condition that did not display DNA integrity loss. This may be regarded as an indication of different threshold limits for expression of toxicity as enzyme inhibition or as DNA damage. In agreement with the results reported by Modesto and Martinez [52,53], who found a decreased activity of antioxidant enzymes (SOD, CAT, GST, and GPX) in fish exposed to Roundup®, the present results point to enzyme inhibition as a potential mechanism through which this herbicide can induce oxidative stress.

Saleha Banu et al. [54] stated that, besides ROS-dependent processes, organophosphate pesticides can cause DNA strand breaks by inhibiting enzymes involved in DNA repair or interacting with DNA. Giving support to this suggestion, organophosphates were presented as alkylating agents [55] that affect DNA bases either directly or indirectly via protein alkylation [56,57]. A study with mice also showed the ability of Roundup® to induce a dose-dependent formation of DNA adducts [58]. Therefore, the previously invoked mechanisms (ROS-independent processes) played a key role in the generation of DNA damage in hepatic cells under short exposures (1 day), while for 3-day exposure (116 μg L^{-1}) oxidation of DNA bases appears as a relevant mechanism of damage.

4.3. Gills versus liver responses

The comparative analysis of both comet assay and antioxidant endpoints in gills and liver following 1-day exposure revealed similar patterns of response and comparable susceptibly towards Roundup-induced genotoxicity. In addition, both organs displayed a remarkable decrease of genetic damage after the 3-day exposure to 58 μg L^{-1}. There was a strong organ-specificity as GDI values in liver returned to the control level whereas in gills they remained significantly higher than the control. This may be an indication of a better adaptive behaviour of hepatic cells, which can be related with a higher capacity to maintain the genomic stability by detecting and repairing damaged DNA. This fact makes gills more adequate for genotoxic risk assessment in environmental waters in the presence of moderate waterborne concentrations of this herbicide. Another difference between the two organs concerned the time-related increase in GDI levels, which was only observed in liver (116 μg L^{-1}).

Under the test conditions, the antioxidant system seems to be more responsive in gills, also showing lesser vulnerability to enzyme inhibition compared with liver.

In general, the variation in the preponderance of ROS-dependent processes as a function of concentration and time did not show any organ-specificity.

5. Conclusions

The present findings clearly demonstrate the genotoxic properties of Roundup® expressed as DNA strand-breaks (measured by the comet assay) in gills and liver cells of *A. anguilla* exposed to realistic concentrations of this herbicide. This result is indicative of a risk to fish populations resulting from the occurrence of this agrochemical in natural water bodies.

The investigation of the causative involvement of oxidative stress demonstrated that the type of DNA damage varies with the

test concentration and exposure duration. After the 1-day exposure, an increase in pro-oxidant state is not a necessary condition for the induction of DNA-damaging effects of Roundup®. Nevertheless, by increasing the exposure duration to 3 days, ROS-dependent processes gained preponderance as a mechanism of DNA damage in the higher concentration (116 $\mu g\,L^{-1}$), as evidenced by the antioxidant activation observed in gills and the net increase in FPG-sensitive sites (indicating the presence of oxidatively altered DNA bases) detected in liver by means of the enzyme-modified comet assay.

Overall, the mechanisms involved in Roundup-induced DNA damage seem to be similar in both organs. However, liver showed to be less susceptible to DNA integrity loss at the lower concentration (58 $\mu g\,L^{-1}$).

6. Ethical statement

This study was conducted in accordance with national guidelines (Portaria no. 1005/92 de 23 Outubro) for the protection of human subjects and animal welfare.

7. Conflicts of interest

The authors declare that there are no conflicts of interests.

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