Mutagenesis vol. 25 no. 5 pp. 523–530, 2010
Advance Access Publication 19 July 2010
doi:10.1093/mutage/geq038

European eel (*Anguilla anguilla*) genotoxic and pro-oxidant responses following short-term exposure to Roundup®—a glyphosate-based herbicide

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Received on January 18, 2010; revised on May 28, 2010; accepted on June 14, 2010

The glyphosate-based herbicide, Roundup®, is among the most used pesticides worldwide. Due to its extensive use, it has been widely detected in aquatic ecosystems representing a potential threat to non-target organisms, including fish. Despite the negative impact of this commercial formulation in fish, as described in literature, the scarcity of studies assessing its genotoxicity and underlying mechanisms is evident. Therefore, as a novel approach, this study evaluated the genotoxic potential of Roundup® to blood cells of the European eel (*Anguilla anguilla*) following short-term (1 and 3 days) exposure to environmentally realistic concentrations (58 and 116 μg/l), addressing also the possible association with oxidative stress. Thus, comet and erythrocytic nuclear abnormalities (ENAs) assays were adopted, as genotoxic end points, reflecting different types of genetic damage. The pro-oxidant state was assessed through enzymatic (catalase, glutathione-*S*-transferase, glutathione peroxidase and glutathione reductase) and non-enzymatic (total glutathione content) antioxidants, as well as by lipid peroxidation (LPO) measurements. The Roundup® potential to induce DNA strand breaks for both concentrations was demonstrated by the comet assay. The induction of chromosome breakage and/or segregational abnormalities was also demonstrated through the ENA assay, though only after 3-day exposure to both tested concentrations. In addition, the two genotoxic indicators were positively correlated. Antioxidant defences were unresponsive to Roundup®. LPO levels increased only for the high concentration after the first day of exposure, indicating that oxidative stress caused by this agrochemical in blood was not severe. Overall results suggested that both DNA damaging effects induced by Roundup® are not directly related with an increased pro-oxidant state. Moreover, it was demonstrated that environmentally relevant concentrations of Roundup® can pose a health risk for fish populations.

Introduction

The use of pesticides has become essential to control pests in modern agriculture, contributing significantly to enhance its productivity. Among pesticides, herbicides are the most dangerous for aquatic environment because they easily reach the water bodies, mainly through soil surface run-off (1). Compounds based on glyphosate are broad-spectrum non-selective organophosphate herbicides and the most extensively used worldwide (2). The herbicide Roundup® is a commercial formulation which contains glyphosate [*N*-(phosphonomethyl) glycine] as the active ingredient and polyethoxylene amine as surfactant. In natural water bodies, Roundup® (measured as glyphosate acid equivalents) has been detected at concentrations from 0.01 to 0.7 mg/l (3,4), reaching 1.7 mg/l in an extreme situation after direct application to water (5). Despite the acute toxicity of glyphosate was considered to be low (6,7), glyphosate-based commercial formulations are generally more toxic than pure glyphosate (8,9) mainly due to the interference of surfactants such as polyethoxylene amine (10).

To understand the impact on aquatic biota of this type of agrochemicals, fish are often used as sentinels, due to their key function in the trophic web, bioaccumulation propensity and responsiveness to low concentrations (11,12). Moreover, fish showed to be more sensitive to Roundup® than terrestrial organisms (13,14) highlighting the ecotoxicological relevance to approach this taxon. Despite the description of a variety of deleterious effects induced by Roundup® in fish (15–18), the scarcity of studies evaluating its genotoxic potential is evident. The few available studies demonstrated the genotoxicity of Roundup® to fish expressed as cytogenetic and DNA-damaging effects (2,14,19). Nevertheless, these studies concerned only tropical species, adopting excessively high concentrations and did not explore any mechanisms behind genetic damage.

Oxidative stress is an imbalance between the production of reactive oxygen species (ROS) and the body's ability to detoxify the reactive intermediates or repair the resulting damage. Subsequently, peroxides and free radicals can damage potentially all the components of the cell, including proteins, lipids, DNA and RNA (20). In addition to damaging DNA, ROS also disrupt the function of DNA repair proteins (21). To counteract ROS-induced damage, cells evolved antioxidant systems as a major defence mechanism. Thus, variations in the antioxidant defences can be very sensitive in revealing a pro-oxidant condition and have been proposed as indicators of pollutant mediated oxidative stress (22,23). Human and mammal studies with pesticides, especially organophosphates, demonstrated that DNA damage and oxidative stress are mechanistically linked (20,24). Though Roundup® induced mild oxidative stress in goldfish (18), the association between oxidative stress and genetic damage remains unidentified in fish.

The central aim of the present study was to evaluate the genotoxic potential of Roundup® to blood cells of fish (*Anguilla anguilla*), following short-term exposure to environmentally realistic concentrations (58 and 116 μg/l), addressing its possible association with oxidative stress. Genotoxic end points such as comet and erythrocytic nuclear abnormalities

(ENAs) assays were adopted, in order to reflect genetic damage at different levels. The comet assay, one of the most commonly used methods in environmental toxicology and successfully applied to fish for assessing DNA strand breaks and alkali labile sites (25,26), represents an early sign of damage, which might be subject to a repair process. The ENA assay, based on the detection of micronuclei and other nuclear anomalies (27), signals *in vivo* chromosome breakage (clastogenicity) or loss and mitotic spindle apparatus dysfunction (aneugenicity) (28,29). Hence, ENAs are irreparable lesions, representing later and less transient alterations when compared with those detected by the comet assay. Catalase (CAT), glutathione-*S*-transferase (GST), glutathione peroxidase (GPx), glutathione reductase (GR) activities, total glutathione (GSHt) content and levels of thiobarbituric acid reactive substances (TBARS) were determined as indicators of the pro-oxidant state. The concomitant assessment of genotoxic markers and indicators of pro-oxidant state represents an innovative approach in the context of pesticide-induced genotoxicity in fish.

Material and methods

Chemicals

A commercial formulation of glyphosate [*N*-(phosphonomethyl) glycine]—Roundup®—distributed by Bayer CropScience Portugal, containing isopropylammonium salt of glyphosate at 485 g/l as the active ingredient (equivalent to 360 g/l or 30.8% of glyphosate) and polyethoxylene amine (16%) as surfactant, was used. All the other chemicals were obtained from the Sigma–Aldrich Chemical Company (Spain).

Test animals and experimental design

European eel (*A.anguilla* L.) specimens with an average length of 25 ± 3 cm and weight 32 ± 5 g (yellow eel stage) were captured from an unpolluted area of 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, dechlorinated and recirculating tap water, with the following physicochemical conditions: salinity 0, temperature 20 ± 1°C, pH 7.3 ± 0.2, ammonia <0.1 mg/l, dissolved oxygen 8.1 ± 0.5 mg/l.

The experiment was carried out in 20 l aquaria, in a static mode, under the conditions described for the acclimation period. After acclimation, 36 eels were divided into six aquaria (six fish per aquaria; $n = 6$) and exposed to 58 µg/l (two aquaria) and 116 µg/l (two aquaria) of Roundup®, equivalent to 18 and 36 µg/l of glyphosate, respectively. Another two aquaria were kept with clean water—control groups. For each pesticide concentration, 1- and 3-day exposures were tested, corresponding to the two different aquaria previously mentioned. Fish were not fed during experimental period. Fish blood was collected (~400 µl) from the posterior cardinal vein using heparinised Pasteur pipettes. Blood smears were immediately prepared for ENA assay. Two microliters of blood were diluted in 1 ml of phosphate-buffered saline for comet assay. The remainder volume was stored in microtubes and kept at −80°C until further procedures for oxidative stress analyses. Following sampling, fish were sacrificed by cervical transaction.

Evaluation of genetic damage

Comet assay. The alkaline version of the comet assay was performed according to Collins (30) methodology with slight modifications. Two gel replicates, containing each one $\sim 2 \times 10^4$ cells (using the whole blood previously diluted in phosphate-buffered saline) in 70 µl of 1% low melting point agarose in phosphate-buffered saline, were placed on one glass microscope slide, precoated with 1% normal melting point agarose. The gels were covered with glass coverslips, left for ±5 min at 4°C to solidify agarose and then immersed in a lysis solution (2.5 M NaCl, 0.1 M ethylenediaminetetraacetic acid, 10 mM Tris and 1% Triton X-100, pH 10) at 4°C, for 1 h. Slides were immediately processed according to the conventional comet assay (30). Briefly, slides were gently placed in the electrophoresis tank, immersed in electrophoresis solution (±20 min) to alkaline treatment. DNA migration was performed at a fixed voltage of 25 V, a current of 300 mA, which results in 0.7 V/cm (achieved by adjusting the total volume of buffer). The slides were stained with ethidium bromide (20 µg/ml). One slide with two gels (100 nucleoids per gel) was observed for each fish using 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 index (GDI) was calculated multiplying the mean percentage of nucleoids in each class by the corresponding factor, according to this formula:

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

Results were expressed as 'arbitrary units' in a scale of 0–400 per 100 scored nucleoids (as average value for the two gels observed per fish). Besides the GDI, the frequency of nucleoids observed in each comet class was also expressed as recommended by Azqueta *et al*. (31).

ENA assay. This assay was carried out in mature peripheral erythrocytes according to the procedure of Pacheco and Santos (32). Briefly, one blood smear per animal was fixed with methanol during 10 min and stained with Giemsa (5%) during 30 min. From each smear, 1000 erythrocytes were scored under ×1000 magnification to determine the frequency of the following nuclear lesion categories: kidney shaped nuclei (K), lobed nuclei (L), binucleate or segmented nuclei (S) and micronuclei (MN). In addition, notched nuclei (N) were also scored as suggested by Fenech (28) and Ayllon and Garcia-Vazquez (33). Final results were expressed as the mean value (‰) of the sum for all the lesions observed (K + L + S + N + MN).

Biochemical analyses

Tissue preparation and fractionation. Whole-blood samples were lysed through homogenisation in a 1:15 ratio (blood volume:buffer volume), using a Potter–Elvehjem homogeniser, in chilled phosphate buffer (0.2 M, pH 7.4). This lysate was then divided into three aliquots: for TBARS and GSHt quantification, as well as for post-mitochondrial supernatant (PMS) preparation to be used in the enzymatic determinations. The PMS fraction was obtained by centrifugation (Eppendorf 5415R centrifuge) at 13 400 g for 20 min at 4°C. Aliquots of PMS were stored in microtubes at −80°C until analyses.

Measurement of antioxidant responses and peroxidative damage. CAT activity was assayed (at 25°C) by the method of Claiborne (34) as described by Giri *et al*. (35). Change in absorbance was recorded spectrophotometrically at 240 nm and CAT activity was calculated in terms of micromoles H_2O_2 consumed per minute per milligram of protein ($\varepsilon = 43.5$/M·cm).

GST activity was determined (at 25°C) using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate, according to the method of Habig *et al*. (36). Absorbance was recorded spectrophotometrically at 340 nm for 3 min. The enzyme activity was calculated as nanomoles CDNB conjugate formed per minute per milligram of protein ($\varepsilon = 9.6$/mM·cm).

GPx activity was determined (at 25°C) according to the method of Mohandas *et al*. (37). NADPH oxidation was recorded spectrophotometrically at 340 nm and GPx activity was calculated in terms of nanomoles NADPH oxidised per minute per milligram of protein ($\varepsilon = 6.22 \times 10^3$/M·cm).

GR activity was assayed (at 25°C) by the method of Cribb *et al*. (38). The assay determines indirectly the GR activity by measuring the NADPH disappearance associated to the reduction of oxidised glutathione catalysed by GR. Change in absorbance was registered spectrophotometrically at 340 nm during 3 min and GR activity calculated as nanomoles of NADPH oxidised per minute per milligram of protein ($\varepsilon = 6.22 \times 10^3$/M·cm).

For GSHt quantification, protein content in the tissue lysate was precipitated with trichloroacetic acid (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 reduced glutathione reacts with 5,5, dithiobis-tetranitrobenzoic acid and produces a yellow coloured 5-thio-2-nitrobenzoic acid (TNB). The rate of TNB production is proportional to the concentration of glutathione in the sample (39). Formation of TNB was measured by spectrophotometry at 412 nm and the results expressed as nanomoles TNB formed per minute per milligram of protein ($\varepsilon = 14.1$/mM·cm).

As estimation of lipid peroxidation (LPO), TBARS quantification was carried out in the previously prepared lysate (treated with 4% of 1-1 butylated hydroxytoluene in methanol to prevent oxidation) as adapted by Filho *et al*. (40). The absorbance was measured at 535 nm and the rate of LPO was expressed in nanomoles of TBARS formed per milligram of fresh tissue ($\varepsilon = 1.56 \times 10^5$/M·cm).

Total protein contents were determined according to the Biuret method (41), using bovine serum albumin as a standard.

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 analysis of variance was used to compare the different treatments within the same exposure duration as well as to compare the same treatment in different exposure durations. The Tukey's test was applied for post-hoc comparison. Whenever the assumptions for parametric statistics failed, the non-parametric correspondent test (Kruskall–Wallis) was performed, followed by the non-parametric all pairwise multiple comparison procedure (Dunn's test) (42). Differences between means were considered significant when $P < 0.05$. The relationship between the assessed parameters was explored using linear regression analyses. The correlation coefficient (r) was calculated and its statistical significance (P) was determined from the table of critical values for the correlation coefficient (42).

Results

DNA damage as comet assay

Fish exposed to Roundup® revealed a significantly higher DNA damage, measured as GDI, in both concentrations after 1 and 3 days (Figure 1), when compared to the respective control group. Concerning 1-day exposure, fish exposed to Roundup® presented a 1.2 and 1.7 times increase, respectively, for 58 and 116 μg/l compared with the control. An increase of 1.4 times was observed comparing Roundup® concentrations; nonetheless, no significant differences were observed between these two exposed groups. Similar results were observed after 3-day exposure as exposed groups displayed damage increments of 1.4 and 1.7 times, respectively, for 58 and 116 μg/l Roundup®. The difference between exposed groups was less pronounced (1.2 times increase for 116 μg/l) than that observed after 1-day exposure. Globally, the GDI results were concentration dependent, whereas no time-related alterations were noticeable.

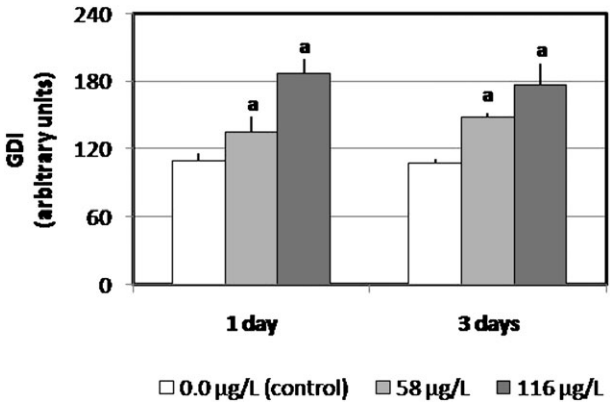

Fig. 1. Mean GDI (expressed as arbitrary units), measured by comet assay, in peripheral blood cells of *A.anguilla* exposed to 58 and 116 μg/l Roundup®, during 1 and 3 days. Letter 'a' denotes statistically significant differences ($P < 0.05$) versus control. Bars represent the standard error.

Individual DNA damage classes (Table I), unlike the pattern displayed in total DNA damage (GDI; Figure 1), revealed a time-related difference concerning the magnitude of damage. After the first day, significant differences were found in classes 1–4 between fish exposed to 116 μg/l Roundup® and control, whereas fish exposed to 58 μg/l Roundup® showed significant increases only in classes 1 and 2. Following 3-day exposure, classes 1–3 demonstrated significantly higher values, comparing to control, in both Roundup® concentrations. Overall, considering both Roundup® concentrations and both exposure times, classes 1 and 2 were the most prevalent classes of damage.

The comparison between exposure lengths (within the same treatment) showed no differences in terms of GDI and a significant decrease in class 2 frequency from 1- to 3-day exposure in the 58 μg/l group.

ENA frequency

No significant alterations were found in ENA frequency following the first day of exposure (Figure 2). However, an increased tendency was perceptible in both Roundup® concentrations, relatively to control. A similar pattern was observed when each lesion category was considered individually (Table II). Nevertheless, significant ENA increases were found after 3-day exposure in both exposed groups in comparison with the control. In addition, a significant ENA increase (around two times) was observed from 58 to 116 μg/l Roundup® group. The individual analysis of each nuclear lesion category revealed that K and L frequencies were significantly higher in both exposed groups after 3-day exposure. These categories were also significantly higher in fish exposed to 116 μg/l Roundup® when compared to 58 μg/l. Lobed nuclei was the most commonly detected abnormality in fish exposed for 3 days to 58 μg/l Roundup® (L > K > S > N = MN), whereas in 116 μg/l group, the highest frequency was registered for K (K > L > S > N = MN). The sub-total K + L + S + N displayed a pattern similar to that one observed for total ENA frequency.

Comparing results between exposure lengths, a significant decrease was observed from 1- to 3-day exposure in control group in terms of L (Table II) and total ENA (Figure 2) frequencies, while in the 116 μg/l group, a significant increase was observed in K frequency.

Antioxidant responses and lipid peroxidative damage

Concerning antioxidant responses measured in both Roundup®-treated groups (Figures 3A–E), no significant alterations were observed after 1- and 3-day exposures. However, the evaluation of peroxidative damage (Figure 3F) showed a significant increase in TBARS values after 1-day

Table I. Mean frequency of each DNA damage class (± standard error), measured by comet assay, in peripheral blood cells of *A.anguilla* exposed to 58 and 116 μg/l Roundup®, during 1 and 3 days.

		Damage classes				
Exposure time	Roundup® concentration (μg/l)	0	1	2	3	4
1 day	0.0 (control)	0.00 ± 0.00	81.60 ± 5.73	17.30 ± 5.07	1.10 ± 0.78	0.00 ± 0.00
	58	1.40 ± 1.40	44.30 ± 6.93[a]	45.30 ± 3.01[a]	7.30 ± 4.07	1.70 ± 1.06
	116	2.00 ± 1.52	31.40 ± 4.10[a]	48.50 ± 2.41[a]	13.60 ± 2.66[a]	4.50 ± 1.93[a]
3 days	0.0 (control)	0.00 ± 0.00	93.00 ± 3.43	6.60 ± 3.22	0.40 ± 0.24	0.00 ± 0.00
	58	0.00 ± 0.00	59.00 ± 2.24[a]	34.10 ± 1.07[a,♦]	6.80 ± 1.91[a]	0.10 ± 0.10
	116	0.00 ± 0.00	41.90 ± 10.58[a]	41.80 ± 4.51[a]	13.80 ± 5.66[a]	2.50 ± 1.47

Statistically significant differences ($P < 0.05$) are 'a' versus control and '♦' versus 1-day exposure (for the same exposure condition).

Table II. Mean frequency (‰) of each nuclear abnormality category (± standard error) in peripheral erythrocytes of *A.anguilla* exposed to 58 and 116 µg/l Roundup®, during 1 and 3 days.

		Nuclear abnormality categories					
Exposure time	Roundup® concentration (µg/l)	Kidney shaped (K)	Lobed (L)	Segmented (S)	Notched (N)	Sub-total (K + L + S + N)	MN
1 day	0.0 (control)	8.60 ± 1.69	12.20 ± 3.22	0.60 ± 0.24	0.00 ± 0.00	21.40 ± 3.98	0.60 ± 0.40
	58	11.20 ± 2.11	19.00 ± 2.12	2.00 ± 0.95	0.00 ± 0.00	32.20 ± 4.79	0.20 ± 0.20
	116	13.40 ± 2.27	22.20 ± 3.50	1.00 ± 0.55	0.00 ± 0.00	36.60 ± 4.43	0.80 ± 0.49
3 days	0.0 (control)	4.40 ± 1.44	3.20 ± 1.32$^{\blacklozenge}$	0.40 ± 0.40	0.00 ± 0.00	8.00 ± 1.82$^{\blacklozenge}$	0.00 ± 0.00
	58	12.40 ± 2.54^{a}	14.00 ± 1.92^{a}	0.40 ± 0.40	0.00 ± 0.00	26.80 ± 3.56^{a}	0.00 ± 0.00
	116	28.00 ± 1.92$^{a,b,\blacklozenge}$	20.60 ± 1.08^{a,b}	0.80 ± 0.80	0.00 ± 0.00	49.40 ± 2.89$^{a,b,\blacklozenge}$	0.00 ± 0.00

Statistically significant differences ($P < 0.05$) are 'a' versus control, 'b' versus 58 µg/l (within the same exposure duration) and '♦' versus 1-day exposure (for the same exposure condition).

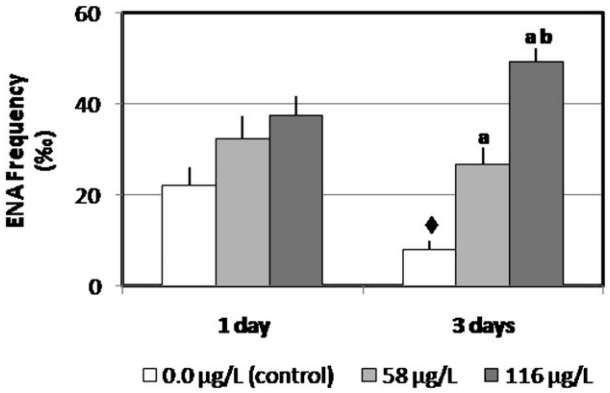

Fig. 2. Mean frequency (‰) of ENAs in *A.anguilla* exposed to 58 and 116 µg/l Roundup®, during 1 and 3 days. Statistically significant differences ($P < 0.05$) are 'a' versus control and 'b' versus 58 µg/l (within the same exposure duration); '♦' versus 1-day exposure (for the same exposure condition). Bars represent the standard error.

exposure to 116 µg/l Roundup®, when compared to control as well as to 58 µg/l groups. In spite of the previous results, no LPO increase was observed after 3-day exposure. The comparison between 1- and 3-day exposures revealed significant LPO increases in control and 58 µg/l groups.

Correlations between biological parameters

The correlation between all biological parameters was statistically tested by analysing the data obtained after 1- and 3-day exposures to 58 and 116 µg/l Roundup®. However, only the correlation between DNA damage and ENA frequency was statistically significant (Figure 4).

Discussion

A model to estimate the worst-case exposure conditions was developed by Giesy *et al*. (13) who set 0.271–0.406 and 0.339–0.677 mg/l of Roundup® as the maximum concentrations likely to be found in surface waters following terrestrial uses and direct application to water, respectively. These estimations have proven to be correct, though higher levels were sporadically found (5). In this perspective, the Roundup® concentrations tested in the present study (58 and 116 µg/l) are realistic, mainly in the context of agriculture applications (the primary use of Roundup®), representing an important progress in relation to previous fish studies (2,19) where the adopted concentrations were one order of magnitude higher.

As a novel approach in the context of pesticide genotoxicity assessment in fish, the current research encompassed the evaluation of genotoxic end points with the evaluation of pro-oxidant state, a putatively decisive condition on determining the extent and type of genetic damage.

Fish erythrocytes have been proposed as a tool for studying several aspects of toxicology (32,43). Fish erythrocytes are considered as a major site for ROS production due to their role in the oxygen transport via hemoglobin as wells as due to oxygen utilisation. Moreover, since toxic chemicals are absorbed and then transported through the bloodstream, they contact directly with the erythrocytes, which in turn are among the first cells to suffer toxic effects (44).

Genotoxic damage induced by Roundup®

Analysing comet results, the Roundup® potential to induce DNA strand breaks in blood cells became clear, as both concentrations showed increased GDI values after 1- and 3-day exposure. In spite of a perceptible concentration dependence, no clear time-related variations were identified on the basis of GDI values. Though a general pattern marked by the prevalence of classes 1 and 2 could be identified, concentration- and time-dependent alterations were better discernible when the five classes were analysed individually. Hence, after 1-day exposure only 116 µg/l Roundup® induced significant increases of classes 3 and 4. Moreover, after 3-day exposure, the frequency of class 4 was no more significantly different from the control. Fish exposed to 58 µg/l presented a prevalence of class 2 on day 1, whereas on day 3, the peak was observed for class 1, revealing a time-related attenuation of the effect. The same pattern was perceptible for 116 µg/l, though not so extensive, which by itself is also indicative of a concentration-dependence. Therefore, it should be inferred that comet results presentation displaying the values for each damage class can offer detailed additional information, whereas its collective analysis (GDI) may mask some variations.

The main outcome of current comet assay is in agreement with data reported by Çavas and Könen (19) and Cavalcante *et al*. (2) in tropical fish species, where the Roundup® potential to affect DNA integrity in blood cells was also observed. However, the time-related variation seemed to be clearly affected by species and/or exposure concentrations. *Prochilodus lineatus* exposed to 10 mg/l for 1 day (without test water renewal) exhibited significantly elevated comet scores after 6-h exposure, returning to control levels 18 h later (2). This pattern differs from the present study that shows elevated comet scores up to 3-day exposure, though its time-related attenuation was also observed. This can be explained by the lowering levels of the pesticide (or their metabolites) in blood, combined with the intervention of DNA-repair system and/or heavily damaged

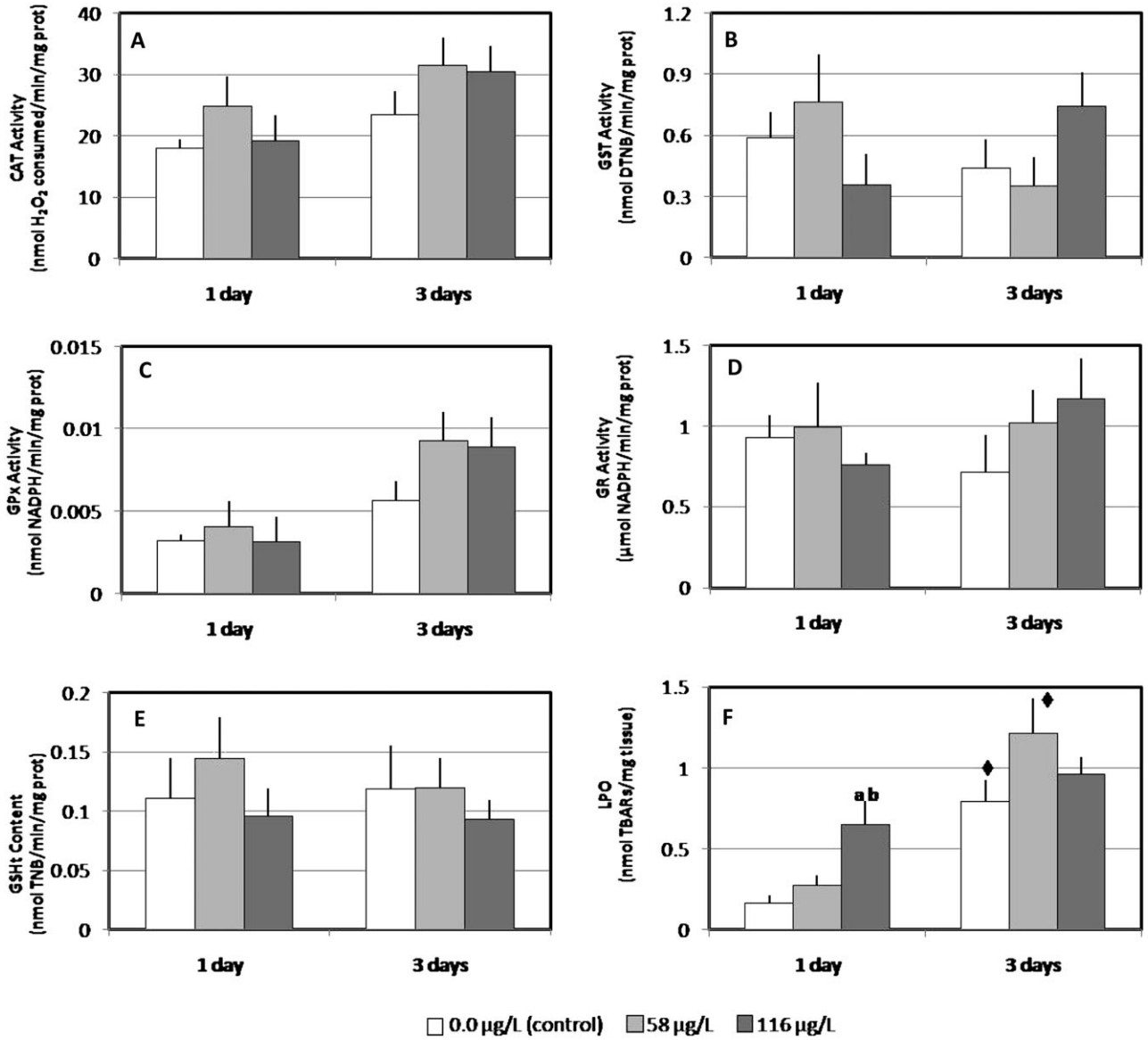

Fig. 3. Mean CAT (**A**), GST (**B**), GPx (**C**) and GR (**D**) activities, as well as GSHt content (**E**) and LPO levels (**F**) in peripheral blood of *A.anguilla* exposed to 58 and 116 µg/l Roundup®, during 1 and 3 days. Statistically significant differences ($P < 0.05$) are 'a' versus control and 'b' versus 58 µg/l (within the same exposure duration); '♦' versus 1-day exposure (for the same exposure condition). Bars represent the standard error.

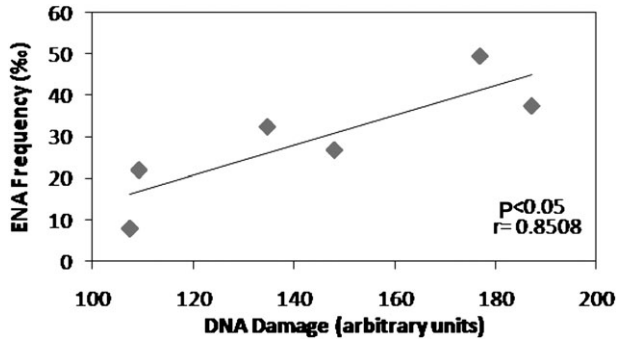

Fig. 4. Correlation between DNA damage (measured by comet assay) and ENA frequency in peripheral blood of *A.anguilla* exposed to 58 and 116 µg/l Roundup®, during 1 and 3 days. Statistical significance and correlation coefficient are represented by *P* and *r*, respectively.

cells catabolism by the spleen. These processes were previously presented by Saleha Banu *et al*. (45) to explain reductions in comet tail-length after 48 and 72 h and a return to control levels after 96 h in blood cells of fish (*Tilapia mossambica*) exposed to an organophosphate pesticide. Moreover, an increased splenic erythrophagia was associated to intense genetic damage in *A.anguilla* (46).

Considering ENA assay, the Roundup® capacity to cause chromosome breakage and/or chromosome segregational abnormalities was demonstrated after 3-day exposure to both tested concentrations. The genotoxic potential identified is in agreement with the study carried out by Çavas and Könen (19) with *Carassius auratus*, though the concentrations tested by these researchers were substantially higher (5, 10 and 15 mg/l). Nevertheless, on that previous study, the lowest concentration required 6 days to induce ENA increase, highlighting the highest sensitivity of *A.anguilla* as a model species for genotoxicity evaluation by ENA assay. In addition, this species specificity is reinforced by the absence of significant responses reported in *P.lineatus* exposed to 10 mg/l for 6, 24 and 96 h (2).

The comparative analysis of comet and MN (or ENA) assays in terms of their sensitivity is a controversial matter. In this

perspective, current ENA data reflected a delayed appearance of damage (in relation to comet assay), indissociable from the need of the exposed cell population to undergo at least one cell cycle (47), which is not a requisite for comet assay. Subsequently, only comet assay showed the ability to detect genetic damage on first day of exposure, confirming the precocious nature of the damaging events involved. Hence, as demonstrated by Wirzinger *et al.* (48), DNA damage measured by the comet assay appears earlier than do MN and is rather short-lived. On the other hand, ENA, unlike comet assay, demonstrated the ability to distinguish the two tested concentrations. In short, it can be inferred that these two genotoxic end points provide complementary information, allowing a more effective assessment of Roundup® genotoxic effects when jointly applied. Accordingly, Wirzinger *et al.* (48) stated previously that both are non-specific biomarkers which reflect different forms of environmental stress, recommending the application of both tests for the evaluation of the genotoxic potential of surface waters.

The two genotoxic indicators used (GDI values and ENA frequencies) showed to be significantly and positively correlated in the present study. Since comet and ENA assays may reflect different types of genetic damage, they can be determined by different factors. Thus, the association between the two responses is not a consensual issue and both positive (49) and negative (48) correlations have already been observed in fish. The present results seem to support the hypothesis of Russo *et al.* (49) that the MN might be induced by strand breaks in the DNA. A question may arise in the context of the correlation between these two tests applied to blood. Whereas ENA assay only considers mature erythrocytes, comet assay is performed using all blood cell types (mature and immature erythrocytes, leukocytes and thrombocytes). However, this may be regarded as a minor question taking into account the negligible abundance of the other cell types when compared to mature erythrocytes.

Oxidative stress as a potential mechanism of genetic damage

Only recently the effects of Roundup® and/or glyphosate on oxidative stress markers have been addressed in fish (17,18,50). The few available studies provided inconclusive information, due to the variety of species and concentration ranges adopted, as well as the target organs analysed. Thus, Roundup® exposure (2.5–20 mg/l) generally suppressed the activities of superoxide dismutase, GST and GR in brain, kidney and liver of *C.auratus* (18), which was explained by a ROS-induced inactivation. Oppositely, liver CAT activity increased in *C.auratus* (only at 10 mg/l) (18) and *P.lineatus* (7.5–10 mg/l) (50), whereas in *Rhamdia quelen* (0.2–0.4 mg/l), it remained unaltered (17).

To our knowledge, this is the first time that these parameters are evaluated in fish blood following exposure to Roundup®. The present results revealed that neither enzymatic nor non-enzymatic antioxidant defences were substantially affected by the herbicide, and thereby did not provide any evidence of pro-oxidant challenge. Considering the present and previous results, it can be suggested that the modulation of antioxidant responses by Roundup® is a concentration dependent process and thus, the lack of significant alterations currently observed in *A.anguilla* may be explained by tissue specificities and by the low concentrations adopted. These results seem to support the idea that the components of Roundup® do not directly enter redox processes (18) and, under the tested conditions (species/concentrations/exposure time), the threshold limit to cause, for instance, enzyme inhibition was not reached in blood.

The LPO levels measured in *A.anguilla* blood were unaffected by the herbicide treatment and only the high concentration, after 1-day exposure, showed enhanced levels. Despite the tissue-specific differences, Lushchak *et al.* (18) found similar results as Roundup® did not increase the levels of lipid peroxides in *C.auratus* liver and brain. On the other hand, Glusczak *et al.* (17) found that Roundup® treatment reduced LPO in brain, did not affect liver, and enhanced LPO in muscle of *R.quelen*.

Overall, the present results suggest that redox-defence system and peroxidative damage, though interdependent, can follow distinct concentration-dependent patterns. This is in agreement with Ahmad *et al.* (22) who stated that LPO increase cannot be predicted only on the basis of antioxidant variations.

Elevated levels of ROS or depressed antioxidant defences may result in DNA oxidation and increased steady-state levels of unrepaired DNA, which is a well-known process underlying genotoxicity, namely in the context of environmental genotoxicants (30,31). This association has already been demonstrated in humans for organophosphate pesticides (20). Though never assessed, oxidative stress was hypothesised as a possible mechanism for Roundup® genotoxic action in fish (2). In this perspective, the present data suggested that, under the tested conditions, both DNA and chromosomal damage induced by Roundup® in blood cells are not supported by an increased pro-oxidant state. However, this causal relationship cannot be definitively rejected namely in the presence of higher pesticide concentrations. Thus, the assessment of oxidatively altered DNA bases, applying for instance the comet assay with an extra step of digesting the nucleoids with enzymes that specifically recognise oxidised pyrimidines and purines or through the direct quantification of 8-hydroxy-2′-deoxyguanosine in the blood plasma, would be helpful in that direction.

According to Saleha Banu *et al.* (45), besides ROS-dependent processes, organophosphate pesticides can cause DNA strand breaks interacting with DNA or inhibiting enzymes involved in DNA repair. Assuming that the slight time-related attenuation of DNA integrity loss can be indicative that DNA-repair system was not inhibited, the interaction between Roundup® constituents (or metabolites) and DNA appears as the most probable mechanism. Giving support to this suggestion, organophosphate pesticides were presented as alkylating agents (51), affecting DNA bases either directly or indirectly via protein alkylation (52,53).

Conclusions

Present data demonstrated the Roundup® genotoxic potential to blood cells of *A.anguilla* exposed to environmentally realistic concentrations. The herbicide showed the capacity to induce both DNA damage as single strand breaks (measured by comet assay) and cytogenetic effects as chromosome or chromatid breaks or loss (measured by ENA assay). Though correlated, the two adopted genotoxic end points demonstrated complementary aptitudes and thus their jointly application is recommended for the detection of potential environmental genotoxicants.

Antioxidants were unresponsive to Roundup®, despite LPO increase after the first day of exposure to the highest concentration, indicating that oxidative stress caused by this herbicide in blood was not severe. In addition, overall results suggested that an increase on pro-oxidant state is not

compulsory for the induction of both cytogenetic and DNA damaging effects of Roundup®.

The present findings on genotoxic properties of Roundup® point out increased initial risk factors towards the generation of long-term adverse effects (e.g. carcinogenic and reproductive impairments) in fish exposed to environmentally relevant levels of this agrochemical.

Funding

Fundação para a Ciência e Tecnologia (Government of Portugal) through the Ph.D. fellowship (SFRH/BD/42103/2007); Centre for Environmental and Marine Studies.

Acknowledgements

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.

Conflict of interest statement: None declared.

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