Genotoxicity Evaluation of the Herbicide Garlon® and Its Active Ingredient (Triclopyr) in Fish (*Anguilla anguilla* L.) Using the Comet Assay

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ABSTRACT: Triclopyr-based herbicides are broadly used worldwide for site preparation and forest vegetation management. Thus, following application, these agrochemicals can inadvertently reach the aquatic ecosystems. Garlon® is one of the most popular commercial denominations of this group of herbicides, considered as highly toxic to fish, even by its manufacturer. Although DNA is frequently regarded as a target of pesticide toxicity, the genotoxic potential of Garlon® to fish remains completely unknown. Hence, the main goal of this study was to evaluate the genotoxicity of Garlon® and its active ingredient (triclopyr), clarifying the underlying mechanisms. Therefore, the comet assay, implemented as the standard procedure, with an extra step involving DNA lesion-specific repair enzymes (formamidopyrimidine DNA glycosylase and endonuclease III), was used to identify DNA damage in blood cells of *Anguilla anguilla* L. Short-term exposures (1 and 3 days) to Garlon® and triclopyr were carried out, adopting environmentally realistic concentrations (67.6 and 270.5 $\mu g\ L^{-1}$ Garlon® and 30 and 120 $\mu g\ L^{-1}$ triclopyr). The results concerning the nonspecific DNA damage proved the risk of the herbicide Garlon® and its active ingredient triclopyr in both tested concentrations and exposure lengths. In addition, the higher genotoxic potential of the formulation, in comparison with the active ingredient, was demonstrated. When the additional breaks corresponding to net enzyme-sensitive sites were considered, none of the conditions revealed significant levels of oxidative damage. This identification of the genotoxic properties of triclopyr-based herbicides to fish highlights the need to develop less hazardous formulations, as well as the adoption of mitigation measures related to the application of these agrochemicals in the framework of forestry and agriculture sustainable management. © 2014 Wiley Periodicals, Inc. Environ Toxicol 30: 1073–1081, 2015.

Keywords: Garlon®; triclopyr; genotoxicity; DNA lesion-specific repair enzymes; fish

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INTRODUCTION

The proper use of herbicides, affecting only restricted terrestrial areas, is a critical issue on forestry and agriculture sustainable management. Nevertheless, following diffuse applications, these agrochemicals frequently reach the aquatic environment by runoff, soil leaching, aerial drift, or inadvertent overspray. Considering this and the fact that several herbicides have already been found to be toxic to aquatic organisms, this type of contamination may pose a severe environmental risk to aquatic ecosystems (Clements et al., 1997; Relyea, 2005). Thus, keeping in mind the aquatic organisms and ecosystems health, conducting studies concerning on fish inhabiting contaminated areas must be considered as extremely relevant.

Triclopyr-based herbicides, belonging to the class of pyridinecarboxylic acids, are broadly used worldwide for site preparation and forest vegetation management (Kreutzweiser et al., 1995; Wojtaszek et al., 2005). Triclopyr (3,4,6-trichloro-2-pyridinyloxyacetic acid) is an auxin-type compound with a spectrum of weed control and mode of action similar to that of phenoxy herbicides. It is taken up through the roots, stems, and leaf tissues of plants, being transported via symplastic processes, and accumulated in the meristematic regions. Death of triclopyr-sensitive plants usually occurs over a period of 7–14 days (Getsinger et al., 2000). Triclopyr can be present in the commercial formulations either in the form of butoxyethyl ester (TBEE) or triethylamine salt. However, there are substantial differences in toxicity of TBEE and triethylamine salt derivatives, with the former shown to be more toxic in aquatic settings (MMWD, 2008).

In natural environments, TBEE is degraded within a few hours in triclopyr acid (the active ingredient) (McCall et al., 1988), which is supposed to be less toxic than its precursor (Kreutzweiser et al., 1995). Regarding the quantifications already performed in aquatic environments, and due to the rapid degradation of TBEE, it is frequent to consider only the triclopyr occurrence (Getsinger et al., 2000; Petty et al., 2003). Getsinger et al. (2000) found triclopyr levels higher than 2 mg L^{-1} in water bodies near agricultural fields, which exceed the limits recommended by manufacturers (around 1.25 mg L^{-1}) (Xie et al., 2005).

Garlon® is one of the most popular commercial denominations of triclopyr-based herbicides, both in Europe and America. These formulations (containing TBEE) were considered highly toxic to aquatic organisms as demonstrated by the observation of lethal effects on fish (Kreutzweiser et al., 1994) as well as avoidance behavior and growth impairment in amphibians (Wojtaszek et al., 2005). However, only a few sublethal parameters concerning Garlon's adverse effects have been assessed in fish. In this context, Kreutzweiser et al. (1995) reported a growth inhibition in rainbow trout exposed to Garlon®, whereas another work (Janz et al., 1991) found no signs of acute physiological stress (e.g. alterations on plasma glucose and lactate concentrations). Moreover, and considering the usual tendency of manufacturers to underestimate the risk associated with their commercial products, it becomes particularly relevant to point out the information depicted in the product label (Dow Agrosciences, Lusosem, Portugal), classifying Garlon® as highly toxic to fish. Thus, it seems evident the importance to evaluate parameters that can better predict the fish condition following exposure to Garlon®, as suggested by the Canadian Health Department (Health Canada, 1991). Additionally, to the authors' knowledge, only one study was performed concerning the toxicity to fish of the active ingredient of Garlon®, individually (US EPA, 1998).

DNA is a frequent target of pesticides toxicity. According to this statement, it has been shown that the analysis of DNA integrity in aquatic organisms is a highly suitable method for evaluating the impact of environmental genotoxicants, allowing the detection of exposure to low concentrations of contaminants, including pesticides (Scalon et al., 2010). Nonetheless, the genotoxic potential of Garlon® and its active ingredient (triclopyr), as well as the mechanisms behind its possible DNA damaging action, remain completely unknown.

Bearing in mind the knowledge gaps identified above, the main goal of the present research was to assess the genotoxic potential of the herbicide Garlon® and its active ingredient triclopyr in fish. *Anguilla anguilla* was selected as the test organism because of its successful adoption in genotoxicity evaluation (Nigro et al., 2002; Pacheco and Santos, 2002; Guilherme et al., 2010, 2012), as well as because of its ability of reporting chemical status of all categories of water bodies (Belpaire and Goemans, 2007). The triclopyr concentrations used in the present study are more than 10 times lower compared with those referred by Getsinger et al. (2000) (2 mg L^{-1} found in water bodies near agricultural fields). Considering the herbicide application moment (spatial and temporally) as an extreme situation, it would be expectable to found lower concentrations (magnitude of μg L^{-1}). Hence, the choice of tested concentrations was based on this rationale, because no more data were found concerning this issue.

Therefore, the comet assay was used to identify DNA damage in blood cells of *A. anguilla* L., following a short-term exposure to Garlon® and triclopyr, adopting environmentally realistic concentrations. As an attempt to clarify the involved DNA damaging mechanisms, besides the standard procedure, comet assay was carried out with an extra step where nucleoids were incubated with DNA lesion-specific repair enzymes, namely formamidopyrimidine DNA glycosylase (FPG) and endonuclease III (EndoIII). This combined methodology allows the detection of a genotoxic risk resulting from unspecific (alkali-labile sites and single strand breaks including those associated with incomplete excision repair sites) and specific (bases oxidation) DNA damages. Hence, this additional step intended to clarify to what extent the DNA damage induced was of oxidative origin.

MATERIALS AND METHODS

Chemicals

The experiment was conducted using the commercial formulation Garlon®, distributed by Dow Agrosciences, containing triclopyr formulated as a TBEE at a concentration of 480 g L^{-1} (or 44.4%) and kerosene (petroleum distillate) as adjuvant. Triclopyr (3,5,6-trichloro-2-pyridinyloxyacetic acid) was obtained from Sigma-Aldrich Chemical Company (Spain). DNA lesion-specific repair enzymes, namely FPG and EndoIII, were purchased from Professor Andrew Collins (University of Oslo, Norway). All the other chemicals needed to carry out the comet assay were obtained from the Sigma-Aldrich Chemical Company.

Test Animals and Experimental Design

European eel (*A. anguilla* L.) specimens with an average weight 0.25 ± 0.02 g (glass eel stage) were captured at Minho river mouth, Caminha, Portugal. Eels were acclimated to laboratory for 20 days and kept in 20-L aquaria under a natural photoperiod in aerated, filtered, dechlorinated, and recirculating tap water, with the following physicochemical conditions: salinity 0, temperature 20°C ± 1°C, pH 7.1 ± 0.1, nitrate 25 ± 0.4 mg L^{-1}, nitrite 0.04 ± 0.03 mg L^{-1}, ammonia 0.1 ± 0.03 mg L^{-1}, and dissolved oxygen 8.1 ± 0.2 mg L^{-1}. During this period, fish were daily fed with fish roe. The experiment was carried out in 1-L aquaria, in a semistatic mode, under the conditions described for the acclimation period. After acclimation, 120 eels were divided into 10 groups, corresponding to five test conditions and two exposures times (5 × 2). Thus, fish were exposed to 67.6 and 270.5 μg L^{-1} Garlon® (groups G1 and G2, respectively) and 30 and 120 μg L^{-1} triclopyr (groups T1 and T2, respectively). Simultaneously, another group was kept in herbicide-free water, under the same experimental conditions, to perform a negative control (group C). For each test condition, 1 and 3 days exposures were carried out. Water medium in 3-day aquaria was daily renewed (100%).

The exposure concentrations of Garlon® were calculated considering the acid equivalents of triclopyr contained in the formulated product (the active ingredient represents 44.4%). Stock solutions of each agent were prepared (in deionized water) just before addition to exposure water.

To each test group was assigned an abbreviation where the first number represents the exposure duration, the letter represents the agent tested, and the second number represents the concentration (1 for the lower and 2 for the higher). The experiment was carried out using triplicate ($n = 3$) groups of four fish for each condition/time (3 × 4 = 12 fish). Fish were not fed during experimental period.

Following exposure, fish were sacrificed by cervical transection at the postopercular region, and blood collected from the heart using heparinized capillary tubes. Two microliters of blood was immediately diluted in 1 mL of ice-cold phosphate-buffered saline to prepare a cell suspension, which was kept on ice until further procedure.

Evaluation of Genetic Damage

The conventional alkaline version of the comet assay was performed according to the methodology of Collins (2004) as adapted by Guilherme et al. (2010), with the proper adjustments to assay procedure with extra step of digesting the nucleoids with endonucleases. A system of eight gels per slide was adopted, based on a model created by Shaposhnikov et al. (2010), to increase the assay output. Briefly, 20 μL of cell suspension (previously prepared in phosphate-buffered saline) was mixed with 70 μL of 1% low-melting-point agarose, in distilled water. Eight drops of 6 μL of cell suspension were placed onto the precoated slide as two rows of four (four groups of two replicates), without coverslips, containing each gel approximately 1500 cells. The gels were left for ±5 min at 4°C to solidify agarose, 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 1 h. 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.

Three sets of slides were prepared: first set was incubated with (1) FPG, second with (2) EndoIII, which converts oxidized purines and pyrimidines into DNA single strand breaks, respectively (Azqueta et al., 2009), and a third (3) set was incubated only with buffer. Hence, 30 μL of each enzyme (diluted in buffer) was applied in each gel, along with a coverslip, prior to incubation at 37°C for 30 min in a humidified atmosphere. The slides were then gently placed in the electrophoresis tank, immersed in electrophoresis solution (±20 min) for 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^{-1} (achieved by adjusting the buffer volume in the electrophoresis tank). The slides were stained with ethidium bromide (20 μg mL^{-1}).

Fifty nucleoids were observed per gel, 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) (Collins, 2004). The total score expressed as a genetic damage indicator (GDI) was calculated multiplying the percentage of nucleoids in each class by the corresponding factor, according to the formula:

$$\begin{aligned} \text{GDI} = {} & [(\%\ \text{nucleoids class 0}) \times 0] \\ & + [(\%\ \text{nucleoids class1}) \times 1] \\ & + [(\%\ \text{nucleoids class2}) \times 2] \\ & + [(\%\ \text{nucleoids class3}) \times 3] \\ & + [(\%\ \text{nucleoids class4}) \times 4] \end{aligned}$$

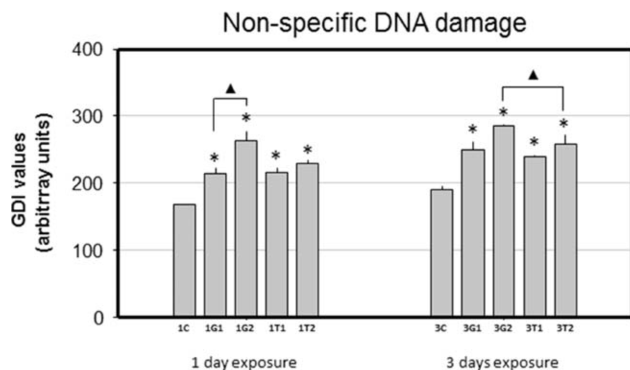

Fig. 1. Mean values of genetic damage indicator (GDI) measured by the standard (alkaline) comet assay in blood cells of *A. anguilla* exposed to 67.6 and 270.5 μg L^{-1} Garlon® (G1 and G2) and 30 and 120 μg L^{-1} triclopyr (T1 and T2), during 1 and 3 days. In the abbreviations for test conditions, the first number represents the exposure duration. Bars represent the standard error. Statistically significant differences ($p < 0.05$) are: (*) in relation to control (C), within the same exposure time and (▲) between treatments, within the same exposure time.

GDI values 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). When the comet assay was performed with additional FPG and EndoIII steps, GDI values were calculated in the same way but the parameter was designated GDI_{FPG} and $GDI_{EndoIII}$, respectively. Additional DNA breaks corresponding to net enzyme-sensitive sites alone (NSS_{FPG} or $NSS_{EndoIII}$) were also expressed. These parameters were calculated based on the difference between GDI_{FPG} and GDI or $GDI_{EndoIII}$ and GDI. Moreover, the frequency of nucleoids observed in each comet class considering GDI_{FPG} and $GDI_{EndoIII}$ was also determined.

Statistical Analysis

Statistica 7.0 software was used for statistical analysis. All data were first tested for normality (Shapiro–Wilk test) and homogeneity of variance (Levene's test) to meet statistical demands. One-way analysis of variance (ANOVA), followed by Dunnett's test as *post hoc* comparison, was applied to compare the treated groups with the control, within the same exposure duration. Three-way ANOVA was applied to test the effect of the factors agent, concentration, and exposure time on the levels of DNA damage, as well as the interactions among them. The Tukey's test was applied as *post hoc* comparison. In all the analyses, differences between means were considered significant when $p < 0.05$ (Zar, 1996).

RESULTS

Nonspecific DNA Damage

Considering GDI values after the first day of exposure, it was possible to notice that all treatments showed to be significantly different from control (Fig. 1). Additionally, a concentration-dependence was perceived when both concentrations of Garlon® (1G1 and 1G2) were compared. In the same way, after 3 days, all treatments exhibited higher GDI levels, when compared with control. At this exposure time, significantly higher levels were found in the higher Garlon® concentration (3G2) compared with the equivalent concentration of its active ingredient (triclopyr) (3T2) (Fig. 1). No time-related differences were detected.

Table I (three-way ANOVA results) revealed a significant effect of the factors agent, concentration, and time on GDI levels, as well as significant interactions agent × concentration and agent × time.

Oxidative DNA Damage

The detection of oxidized bases was achieved by the comet assay with an extra step where nucleoids were incubated with the DNA lesion-specific repair enzymes FPG or EndoIII (Figs. 2 and 3).

TABLE I. Results of three-way ANOVA testing the effect of agent, concentration, and time, as well as the interactions among them on the level of DNA damage in blood cells of *A. anguilla* exposed to Garlon® or triclopyr, during 1 and 3 days

	Factors						Interactions							
	Agent		Concentration		Time		Agent x Concentration		Agent x Time		Concentration x Time		Agent x Time x Concentration	
Parameter	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
GDI	25.82	<0.05	23.38	<0.05	5.96	<0.05	5.35	<0.05	5.96	<0.05	0.39	ns	0.39	ns
GDI_{FPG}	7.51	<0.05	49.41	<0.05	5.88	<0.05	3.37	ns	5.99	<0.05	3.37	ns	18.65	<0.05
NSS_{FPG}	0.14	ns	0.00	ns	11.18	<0.05	1.39	ns	1.17	ns	0.15	ns	1.94	ns
$GDI_{EndoIII}$	9.98	<0.05	31.81	<0.05	8.84	<0.05	7.93	<0.05	0.17	ns	2.52	ns	0.34	ns
$NSS_{EndoIII}$	0.11	ns	0.95	ns	12.20	<0.05	0.14	ns	0.13	ns	0.54	ns	0.58	ns

Both *F* and *p* values are given for each variable. Nonsignificant differences are signalized as "ns".

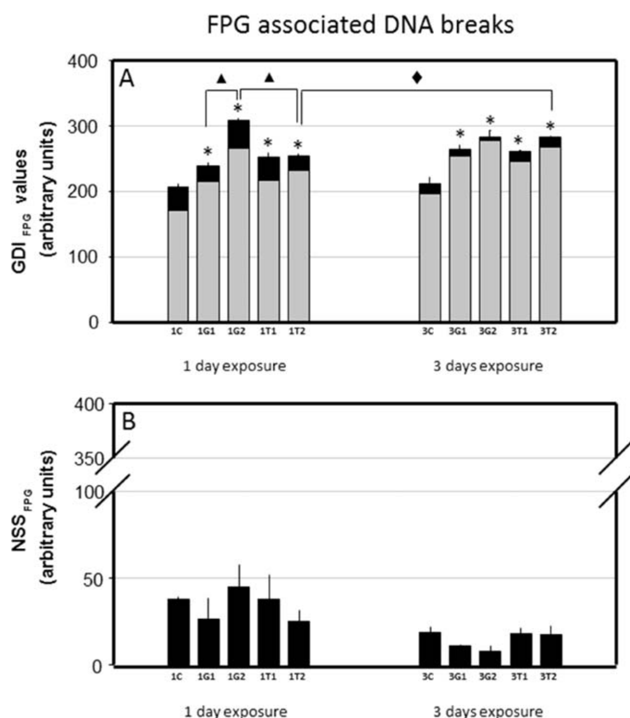

Fig. 2. Mean values of DNA damage, measured by comet assay in blood cells of *A. anguilla* exposed to 67.6 and 270.5 μg L^{-1} Garlon® (G1 and G2) and 30 and 120 μg L^{-1} triclopyr (T1 and T2), during 1 and 3 days. In the abbreviations for test conditions, the first number represents the exposure duration. Values resulted from the assay with an extra step of digestion with formamidopyrimidine DNA glycosylase (FPG) to detect oxidized purine bases: (A) overall damage (GDI_{FPG}) and partial scores, namely genetic damage indicator (GDI; gray) and additional DNA breaks corresponding to net FPG-sensitive sites (NSS_{FPG}; black); (B) NSS_{FPG} alone. Bars represent the standard error. Statistically significant differences ($p < 0.05$) are: (*) in relation to control (C), within the same exposure time; (▲) between treatments, within the same exposure time; and (◆) between exposure times, within the same treatment.

FPG Associated Damage

After 1 day exposure, the digestion with FPG revealed damage levels [GDI_{FPG}; Fig. 2(A)] significantly higher than the control in all treated groups. Moreover, the higher concentration of active ingredient (1T2) displayed significantly lower damage when compared with the equivalent concentration of the commercial formulation (1G2). Like in GDI parameter, GDI_{FPG} was also able to distinguish Garlon® groups, displaying a concentration dependence. As far as the NSS_{FPG} parameter [Fig. 2(B)] is concerned, none of the conditions showed significant differences in relation to the control.

Concerning 3 days exposure [Fig. 2(A)], all treated groups displayed values significantly higher than control. In line with 1 day observations, NSS_{FPG} parameter [Fig. 2(B)] did not show any significant difference. A time-related increase was found concerning the higher concentration of triclopyr (T2) [Fig. 2(A)].

Table I revealed, for GDI_{FPG}, a significant effect of agent, concentration, and time, as well as significant interactions agent × time and agent × time × concentration. NSS_{FPG} only was shown to be significantly affected by time.

To better understand the behavior of the DNA damage depending on the tested agent and exposure length, the damage classes were analyzed individually, considering the GDI_{FPG} parameter (Table II). In general, the significant differences between control and treated groups reflected a pattern similar to that one displayed by the overall score. Furthermore, it can be highlighted that control groups revealed class 2 as the most frequent, whereas triclopyr groups exhibited a prevalence of class 3. Garlon® groups, in

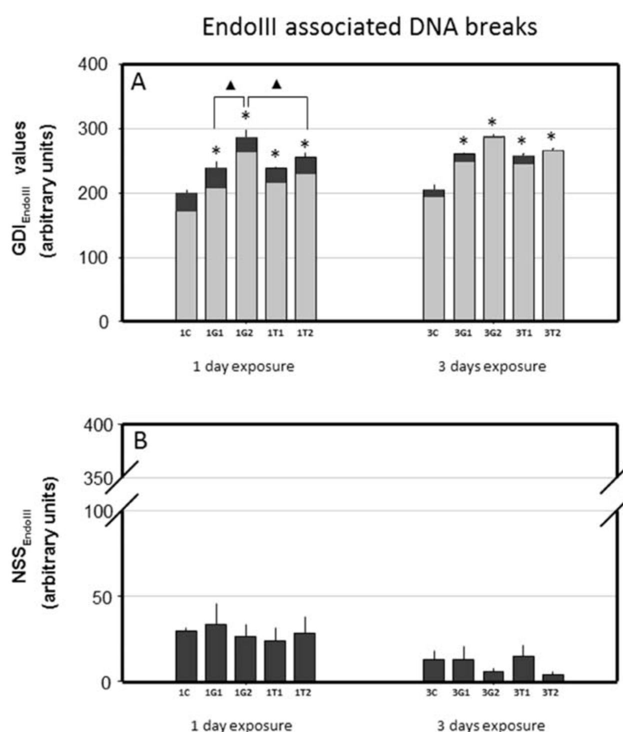

Fig. 3. Mean values of DNA damage, measured by comet assay in blood cells of *A. anguilla* exposed to 67.6 and 270.5 μg L^{-1} Garlon® (G1 and G2) and 30 and 120 μg L^{-1} triclopyr (T1 and T2), during 1 and 3 days. In the abbreviations for test conditions, the first number represents the exposure duration. Values resulted from the assay with an extra step of digestion with endonuclease III (EndoIII) to detect oxidized pyrimidine bases: (A) overall damage ($GDI_{EndoIII}$) and partial scores, namely genetic damage indicator (GDI; light gray) and additional DNA breaks corresponding to net EndoIII-sensitive sites ($NSS_{EndoIII}$; dark gray); (B) $NSS_{EndoIII}$ alone. Bars represent the standard error. Statistically significant differences ($p < 0.05$) are: (*) in relation to control (C), within the same exposure time, and (▲) between treatments, within the same exposure time.

TABLE II. Mean frequencies (%) of damaged nucleoids classes (±standard error), measured by the comet assay including the incubation with the FPG enzyme, in blood cells of *A. anguilla* exposed to 67.6 and 270.5 μg L^{-1} Garlon® (G1 and G2) or 30 and 120 μg L^{-1} triclopyr (T1 and T2), during 1 and 3 days

Exposure Conditions		GDI_{FPG} - DNA Damage Classes				
		0	1	2	3	4
1 day	1C	0.00±0.00	16.88±2.35	59.75±2.86	23.25±5.10	0.13±0.10
	1G1	0.00±0.00	2.25±0.50*	58.61 ±3.43	36.72±3.98	2.42±1.79
	1G2	0.00±0.00	0.00±0.00*	10.92±1.59*[a]	70.00±2.04*[a]	19.08±2.11*[a]
	1T1	0.00±0.00	4.50±1.30*	43.25±4.79*	47.67±4.92*	4.58±1.42
	1T2	0.00±0.00	4.25±0.88*[b]	41.08±2.10*[b]	51.17±2.42*[b]	3.50±1.61[b]
3 days	3C	0.00±0.00	14.13±3.57	59.50±5.51	26.38±9.08	0.00±0.00
	3G1	0.00±0.00	1.06±0.53*	37.00±6.01 *	59.17±5.59*◆	2.78±0.72
	3G2	0.00±0.00	0.17±0.17*	29.18±5.62*	57.51±2.58*	13.14±3.29*[a]
	3T1	0.00±0.00	1_25±0.38*◆	40.00±1.70	54.42±2.75*	4.33±1.17
	3T2	0.00±0.00	0.25±0.25*◆	28.08±1.35*	59.50±1.89*	12.17±1.18*

In the abbreviations for test conditions, the first number represents the exposure duration. Statistically significant differences ($p < 0.05$) are: (*) in relation to control (C), (a) in relation to G1, and (b) in relation to G2, within the same exposure time; and (◆) between exposure times, within the same treatment.

particular, presented classes 2 and 3 as the most representatives. For class 4, a significant frequency increase was only evident for groups corresponding to the highest concentrations of Garlon® (1 and 3 days) and triclopyr (3 days).

EndoIII Associated Damage

Concerning the $GDI_{EndoIII}$ parameter [Fig. 3(A)], all treated groups presented significantly higher values, when compared with control (for both exposure times). Moreover, and specifying for the first day of exposure, it was possible to observe that the active ingredient (1T2) showed a significant decrease when compared with the correspondent concentration of the commercial formulation (1G2). This parameter, as described for GDI and GDI_{FPG}, was able to show a tendency for concentration-dependent increase for Garlon® groups (1G1 and 1G2) [Fig. 3(A)]. $NSS_{EndoIII}$ revealed no significant differences in any of the exposure times [Fig. 3(B)].

Table I displayed significant effects of agent, concentration, and time, as well as interaction agent × concentration for $GDI_{EndoIII}$ parameter. On the other hand, $NSS_{EndoIII}$ parameter, as already observed for NSS_{FPG}, was only affected by the factor time.

In line with what was presented for GDI_{FPG}, the DNA damage classes considering $GDI_{EndoIII}$ were analyzed individually (Table III). Control groups (at both exposure times) displayed class 2 as the most frequent. Treated groups

TABLE III. Mean frequencies (%) of damaged nucleoids classes (±standard error), measured by the comet assay including the incubation with the EndoIII enzyme, in blood cells of *A. anguilla* exposed to 67.6 and 270.5 μg L^{-1} Garlon® (G1 and G2) or 30 and 120 μg L^{-1} triclopyr (T1 and T2), during 1 and 3 days

Exposure Conditions		$GDI_{EndoIII}$ - DNA Damage Classes				
		0	1	2	3	4
1 day	1C	0.00±0.00	19.00±5.31	62.71 ±2.69	18.29±2.62	0.00 ±0.00
	1G1	0.00±0.00	6.67±3.84*	49.78±2.12	41.94±5.63*	1.61 ±0.45
	1G2	0.00±0.00	0.00±0.00*	24.25±6.44*[a]	65.33±2.03*[a]	10.42±4.71
	1T1	0.00±0.00	4.92±1.39*	53.92±2.87	39.67±0.88*	1.50±0.95
	1T2	0.00±0.00	4.33±0.93*	42.00±4.27*[b]	48.08±1.80*[b]	5.58±3.01
3 days	3C	0.00±0.00	17.25±4.90	61.25±3.27	21.50±8.16	0.00±0.00
	3G1	0.00±0.00	1.58±0.42	40.61 ±1.68*	55.58±2.10*◆	2.22±0.26
	3G2	0.00±0.00	0.25±0.14*	25.42±1.23*	60.50±1.25*	13.83±1.58*[a]
	3T1	0.00±0.00	2.00±0.63*	43.75±1.70*	49.08±0.92*	5.17±1.73
	3T2	0.00±0.00	0.17±0.08*	38.67±3.25*	56.58±3.92*	4.58±1.23

In the abbreviations for test conditions, the first number represents the exposure duration. Statistically significant differences ($p < 0.05$) are: (*) in relation to control (C), (a) in relation to G1, and (b) in relation to G2, within the same exposure time; and (◆) between exposure times, within the same treatment.

showed that damage was reflected mostly as class 3, with the exception of 1G1 and 1T1 groups (where class 2 was the most representative). Considering class 4, and contrarily to what was found for GDI_{FPG}, $GDI_{EndoIII}$ was not able to distinguish between treatments, with the exception of Garlon® (3 days exposure).

DISCUSSION

The present study intended to clarify the genotoxic potential of the triclopyr-based herbicide Garlon® and its active ingredient. The adopted approach was based on a previous study performed by the authors, concerning the genotoxicity of another herbicide (Roundup®) and the respective components (Guilherme et al., 2012) where it became clear that DNA damaging patterns followed by the commercial formulation and by the active ingredient (individually) may diverge.

Despite the recognized hazard to aquatic environment of Garlon® and triclopyr, besides the complete absence of genotoxicity evaluation on fish previously highlighted, there is also a scarcity of studies addressing any sublethal effect. Kreutzweiser et al. (1995) assumed the toxicity of TBEE to fish, but, to be precise, what was really assessed was the toxicity of the commercial product Garlon 4® as a whole. Because the mentioned formulation has petroleum distillates in its constitution (namely kerosene), as presented by the manufacturer Dow Agrosciences, the measured effects cannot be strictly attributed to TBEE. Moreover, most studies performed on Garlon 4® toxicity to fish (Janz et al., 1991; Kreutzweiser et al., 1994, 1995) adopted concentrations excessively high (0.25–7.6 mg L^{-1}) (Janz et al., 1991; Kreutzweiser et al., 1995), compared with those that are commonly found in the environment. Therefore, the authors considered it of great importance to carry out the present study adopting environmentally relevant concentrations of the active ingredient (triclopyr) individually (Getsinger et al., 2000; Petty et al., 2003), extrapolating then to the equivalent concentrations of the commercial formulation Garlon®.

In terms of nonspecific DNA damage, the current GDI results proved the genotoxicity of Garlon® and triclopyr, evidencing a dose dependence for the commercial formulation. On the other hand, the difference between the genotoxic potential of both agents call for attention toward a higher adverse effect of the formulation, which might be related to the presence of an adjuvant. It can be also inferred that the formulated product only becomes more genotoxic than the active ingredient (individually) with a continued exposure. Moreover, it was observed that the groups corresponding to the higher concentrations of Garlon® (1G2 and 3G2) were those that exhibited the highest DNA damage extent (as absolute values).

In the context of the assessment of pesticide genotoxicity, the value addition of the use of DNA lesion-specific repair enzymes as an extra step to the standard comet assay has already been demonstrated (Guilherme et al., 2012). Furthermore, this approach also enables the isolation of the oxidative DNA damage. Thus, by analyzing the DNA breaks after the incubation with endonucleases (FPG and EndoIII), the genotoxicity of the tested agents was confirmed (in all treatments and exposure times), and the higher genotoxic potential of Garlon® in relation to triclopyr was reinforced. GDI_{FPG}, in particular, also revealed an increased triclopyr genotoxicity over time, thereby reflecting a higher risk related with its persistence in the aquatic environment.

The analysis of the individual damage classes revealed that either the concentration or the exposure time exerted influence in the magnitude of damage, because higher concentrations and longer time periods presented higher frequencies in class 3. The notorious prevalence of class 3 in Garlon® groups emphasized the hazard caused by this herbicide.

When the additional breaks corresponding to net enzyme-sensitive sites were considered, none of the conditions revealed significant levels of oxidative damage. Hence, NSS_{FPG} and $NSS_{EndoIII}$ parameters were not able, by themselves, to point a considerable damage. Keeping this in mind, the oxidative potential of both tested agents seems to be limited, even though it should not be neglected.

The relative contribution of each component to the overall genotoxicity of herbicide formulations is a matter that remains largely unexplored. Genotoxic studies with fish have been almost exclusively focused on the active ingredients, whereas the effect of adjuvants is frequently ignored. In this framework, a recent study with another commercial herbicide—Roundup®—found that the active ingredient (glyphosate) may be more genotoxic than the formulation, indicating that the surfactant did not contribute to the genotoxicity of the mixture (Guilherme et al., 2012). Considering that Garlon® has kerosene among its constituents (as adjuvant/solvent), the contribution of the latter to the overall effect of the formulation should also be taken into account, because of its recognized toxicity (Arif et al., 1997). However, a USFS (United States Forest Service) report stated that the toxicity of kerosene to aquatic species is approximately 100–1000 folds less than TBEE, suggesting that the acute aquatic toxicity of Garlon® is dominated by this triclopyr precursor (MMWD, 2008). Moreover, Burch and Kline (2007) stated that the toxicity of Garlon® is consistent with the toxicity of TBEE, considering that kerosene does not seem to contribute to the product's toxicity. Nevertheless, though the genotoxicity of kerosene was not evaluated, the present results suggested that the interaction between constituents inside the mixture should not be disregarded, because this solvent increased the impact of the active ingredient triclopyr. Whether this effect represents an additive or a supra-additive (synergistic) interference is an issue that needs further investigation. This outcome is in agreement with Lohani et al. (2000) who found that kerosene can elevate the

genotoxic potential of chrysotile asbestos in hamster embryo fibroblasts. Keeping this in mind, a question emerges concerning the use or development of alternative formulations of triclopyr-based herbicides without kerosene (e.g., Release®) as a better choice to significantly reduce the environmental hazard.

Several studies with pesticides (and its constituents) revealed their genotoxic potential through the use of the standard comet assay (Çavas and Könen, 2007; Sharma et al., 2007; Guilherme et al., 2010). These facts pointed out the subsequent hazard of this kind of contaminants to the aquatic environment, even when low concentrations were considered. The use of comet assay, as a tool in epidemiological studies, allows the early detection of a problem, permitting an efficient intervention to decrease the carcinogenic risk. Accordingly, Au et al. (2010) stated that the exposure to DNA-damaging agents can also affect the components of the vast machinery of DNA repair. This study also point that both damage at DNA and repair machinery contribute to an increase of cancer risk as well as that damage at DNA repair machinery is as deleterious as DNA damage.

Taking all this into account, the authors strongly recommended the inclusion of the DNA integrity evaluation as a useful tool in the ecological risk assessment of water systems contaminated by pesticides.

CONCLUSIONS

Overall, this study revealed, for the first time in fish, the genotoxic potential of the herbicide Garlon® as well as its active ingredient triclopyr. Moreover, the formulation Garlon® showed to be more genotoxic than triclopyr individually. Consequently, the application of alternative formulations of triclopyr-based herbicides without kerosene should be considered in the framework of forestry and agriculture sustainable management.

The ability of exerting oxidative DNA damage could not be demonstrated for any of the tested agents, as depicted in the results as net enzyme-sensitive sites (NSS_{FPG} or $NSS_{EndoIII}$). Although the oxidative potential of both agents seemed to be limited, it should not be completely neglected.

Thus, the present findings on genotoxic properties of the assessed agents call the attention to the hazard to nontarget organisms, namely fish, exposed to these agrochemicals, even when low levels are considered.

This study was conducted in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes, under the supervision of a team member (Mário Pacheco) authorized by the competent authorities. The authors declare that there are no conflicts of interest. They acknowledge the contribution of Eng. Armando Costa, technician at the Department of Biology, University of Aveiro, for his valuable help concerning the knowledge of pesticides use.

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