**Universidade de Aveiro** Departamento de Biologia
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# Risco genotóxico de herbicidas em *Anguilla anguilla* L.

# Genotoxic risk of herbicides to *Anguilla anguilla* L.

Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Doutor Mário Guilherme Garcês Pacheco, Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro e da Doutora Maria Ana Dias Monteiro Santos, Professora Catedrática do Departamento de Biologia da Universidade de Aveiro.

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*“Descobrir como é bom chegar quando se tem paciência.*
*E para se chegar não é preciso dominar a forma, mas a razão.*
*É preciso antes de mais nada querer. Só não conseguimos quando não tentamos.”*
*Amyr Klink*

**o júri**

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**palavras-chave**

Contaminação aquática, herbicidas, genotoxicidade, glifosato, triclopir, stresse oxidativo, *Anguilla anguilla*

**resumo**

No contexto dos contaminantes aquáticos, os herbicidas são considerados como um dos grupos mais perigosos. Uma vez aplicados, estes são facilmente transportados para cursos de água, quer devido a uma pulverização pouco cuidada ou devido a fenómenos de escorrência superficial e/ou subterrânea. A presença destes agroquímicos no ambiente tem vindo a ser associada a efeitos nefastos em organismos não-alvo, como é o caso dos peixes. Contudo, existe ainda uma grande lacuna no que diz respeito à informação científica relacionada com o seu impacto genotóxico. Deste modo, a presente tese foi delineada com o intuito de avaliar o risco genotóxico em peixes de duas formulações de herbicidas: o Roundup®, que tem como princípio activo o glifosato, e o Garlon®, que apresenta o triclopir na base da sua constituição, produtos estes largamente utilizados na limpeza de campos agrícolas, assim como em florestas. Foi ainda planeado desenvolver uma base de conhecimento no que diz respeito aos mecanismos de dano do ADN. Como último objectivo, pretendeu-se contribuir para a mitigação dos efeitos dos agroquímicos no biota aquático, nomeadamente em peixes, fornecendo dados científicos no sentido de melhorar as práticas agrícolas e florestais.

Este estudo foi realizado adoptando a enguia europeia (*Anguilla anguilla* L.) como organismo-teste, e submetendo-a a exposições de curta duração (1 e 3 dias) dos produtos comerciais mencionados, em concentrações consideradas ambientalmente realistas. Para a avaliação da genotoxicidade foram aplicadas duas metodologias: o ensaio do cometa e o teste das anomalias nucleares eritrocíticas (ANE). Enquanto o ensaio do cometa detecta quebras na cadeia do ADN, um dano passível de ser reparado, o aparecimento das ANE revela lesões cromossomais, sinalizando um tipo de dano de difícil reparação. O ensaio do cometa foi ainda melhorado com uma nova etapa que incluiu a incubação com enzimas de reparação (FPG e EndoIII), permitindo perceber a ocorrência de dano oxidativo no ADN. No que diz respeito ao Roundup®, o envolvimento do sistema antioxidante como indicador de um estado pró-oxidante foi também alvo de estudo.

Uma vez que as referidas formulações se apresentam sob a forma de misturas, o potencial genotóxico dos seus princípios activos foi também avaliado individualmente. No caso particular do Roundup®, também foram estudados o seu surfactante (amina polietoxilada; POEA) e o principal metabolito ambiental (ácido aminometilfosfórico; AMPA).

Os resultados obtidos mostraram a capacidade do Roundup® em induzir tanto dano no ADN (em células de sangue, guelras e fígado) como dano cromossómico (em células de sangue). A investigação sobre o possível envolvimento do stresse oxidativo demonstrou que o tipo de dano no ADN varia com as concentrações testadas e com a duração da exposição. Deste modo, com o aumento do tempo de exposição, os processos relacionados com o envolvimento de espécies reactivas de oxigénio (ERO) ganharam preponderância como mecanismo de dano no ADN, facto que é corroborado pela activação do sistema antioxidante observado nas guelras, assim como pelo aumento dos sítios sensíveis a FPG em hepatócitos.

O glifosato e o POEA foram também considerados genotóxicos. O POEA mostrou induzir uma maior extensão de dano no ADN, tanto comparado com o glifosato como com a mistura comercial. Apesar de ambos os componentes contribuirem para a genotoxicidade da formulação, a soma dos seus efeitos individuais nunca foi observada, apontando para um antagonismo entre eles e indicando que o POEA não aumenta o risco associado ao princípio activo.

Deste modo, realça-se a necessidade de regulamentar limiares de segurança para todos os componentes da formulação, recomendando, em particular, a revisão da classificação do risco do POEA (actualmente classificado com "inerte"). Uma vez confirmada a capacidade do principal metabolito do glifosato – AMPA – em exercer dano no ADN assim como dano cromossómico, os produtos da degradação ambiental dos princípios activos assumem-se como um problema silencioso, realçando assim a importância de incluir o AMPA na avaliação do risco relacionado com herbicidas com base no glifosato.

A formulação Garlon® e o seu princípio activo triclopir mostraram um claro potencial genotóxico. Adicionalmente, o Garlon® mostrou possuir um potencial genotóxico mais elevado do que o seu princípio activo. No entanto, a capacidade de infligir dano oxidativo no ADN não foi demonstrada para nenhum dos agentes.

No que concerne à avaliação da progressão do dano após a remoção da fonte de contaminação, nem os peixes expostos a Roundup® nem os expostos a Garlon® conseguiram restaurar completamente a integridade do seu ADN ao fim de 14 dias. No que concerne ao Roundup®, o uso de enzimas de reparação de lesões específicas do ADN associado ao teste do cometa permitiu detectar um aparecimento tardio de dano oxidativo, indicando deste modo um decaimento progressivo da protecção antioxidante e ainda uma incapacidade de reparar este tipo de dano. O período de pós-exposição correspondente ao Garlon® revelou uma tendência de diminuição dos níveis de dano, apesar de nunca se observar uma completa recuperação. Ainda assim, foi evidente uma intervenção eficiente das enzimas de reparação do ADN, mais concretamente as direccionadas às purinas oxidadas.

A avaliação das metodologias adoptadas tornou evidente que o procedimento base do ensaio do cometa, que detecta apenas o dano não-específico no ADN, possui algumas limitações quando comparado com a metodologia que incluiu a incubação com as enzimas de reparação, uma vez que a última mostrou reduzir a possibilidade de ocorrência de resultados falsos negativos. Os dois parâmetros adoptados (ensaio do cometa e teste das ANE) demonstraram possuir aptidões complementares, sendo assim recomendado a sua utilização conjunta com vista a efectuar uma avaliação mais adequada do risco genotóxico.

Globalmente, os resultados obtidos forneceram indicações de grande utilidade para as entidades reguladoras, contribuindo ainda para a (re)formulação de medidas de conservação do ambiente aquático. Neste sentido, os dados obtidos apontam para a importância da avaliação de risco dos herbicidas incluir testes de genotoxicidade. A magnitude de risco detectada para ambas as formulações adverte para a necessidade de adopção de medidas restritivas em relação à sua aplicação na proximidade de cursos de água. Como medidas mitigadoras de impactos ambientais, aponta-se o desenvolvimento de formulações que incorporem adjuvantes selecionados com base na sua baixa toxicidade.

**keywords**

Aquatic contamination, herbicides, genotoxicity, glyphosate, triclopyr, oxidative stress, *Anguilla anguilla*

**abstract**

Herbicides are considered among the most hazardous contaminants of water bodies, since they easily reach these ecosystems through aerial spray drift, artificial drainage systems and surface or sub-surface runoff. The occurrence of these agrochemicals in the aquatic environment has been associated to deleterious effects in non-target organisms, namely fish. However, a considerable gap is evident regarding the scientific information on their genotoxic impact. Therefore, the present thesis was designed with the intention to evaluate the genotoxic risk to fish of the herbicide formulations Roundup® (glyphosate-based) and Garlon® (triclopyr-based), representing broadly used products worldwide to manage unwanted vegetation in agriculture and forestry. It was also planned to develop of a biologically base knowledge on DNA damage mechanisms. As ultimate goal, it was intended to contribute to mitigate the effects of agrochemicals in aquatic biota, namely fish, providing scientific data able to improve forestry and agriculture managing practices.

The study was carried out adopting the European eel (*Anguilla anguilla* L.) as test organism and performing short-term exposures (1 and 3 days) to environmentally realistic concentrations of the mentioned commercial products. Two different genotoxic endpoints were adopted: comet and erythrocytic nuclear abnormalities (ENA) assays. The comet assay measures DNA stand breaks, a repairable type of damage, whereas the ENA assay identifies chromosomal lesions, signalizing a type of damage hardly repairable. The comet assay was also upgraded with an extra-step involving incubation with repair enzymes (FPG and EndoIII), in order to detect oxidative DNA damage. In what concerns to Roundup®, the involvement of the antioxidant system as indication of a pro-oxidant status was also assessed.

Once the aforementioned formulations are presented as mixtures of chemicals, the genotoxic potential of their active ingredients individually was also assessed. In the case of Roundup®, the evaluation of the risk associated to the surfactant (polyethoxylated amine; POEA) and to the major environmental breakdown product of the active principle (aminomethylphosphonic acid; AMPA) was carried out as well.

The results obtained showed the Roundup® ability to induce both DNA (in blood, gills and liver cells) and chromosomal damage (in blood cells). The investigation on the causative involvement of oxidative stress demonstrated that the type of DNA damage varies with tested concentrations and exposure duration. Thus, ROS-dependent processes gained preponderance as a mechanism of DNA damage with the increase of exposure length, which was corroborated by the antioxidant activation observed in gills as well as the net FPG-sensitive sites elevation detected in liver.

Glyphosate and the surfactant POEA were also found to be genotoxic. Moreover, POEA induced the highest extent of DNA damage, when compared to glyphosate and the commercial mixture. Though both components showed to contribute to the overall genotoxicity of the herbicide formulation, the sum of their individual effects was never observed, pointing out an antagonistic interaction between them, indicating that POEA does not increase the risk associated to the active ingredient. These findings also emphasized the need to define regulatory thresholds for all the formulation components, recommending, in particular, the revision of the hazard classification of POEA (classified as “inert” until date). Since the ability of the main environmental metabolite of glyphosate - AMPA - in exert DNA and chromosomal damage was also confirmed, it was pointed out the silent problem that the products of environmental degradation of the active ingredients can constitute. In addition, the importance to include AMPA in risk assessment studies concerning the glyphosate-based herbicides was highlighted.

The formulation Garlon® and its active ingredient triclopyr also showed a clear genotoxic potential. In addition, it was demonstrated the higher genotoxicity of the formulation, in comparison to the active ingredient. However, their ability in exert oxidative DNA damage could not be demonstrated.

In what concerns to the evolution of the damage progression after removal of the contamination source, neither fish exposed to Roundup® nor Garlon® achieved a complete restoration of DNA integrity in 14 days. In relation to Roundup®, the use of the DNA lesion-specific repair enzymes allowed understanding the occurrence of a late oxidative DNA damage, indicating a progressive decay of cell antioxidant protection as well as the incapacity to repair this particular type of damage. The Garlon® post-exposure period revealed a tendency to decrease damage levels, although not enough to be regarded as an effective recovery. However, an efficient intervention of DNA repair enzymes specifically directed to oxidized purines became evident.

Evaluating the performance of the adopted genotoxic endpoints, it was evident that the standard comet procedure, detecting only non-specific DNA damage, displayed some limitations when compared to the methodology that includes the incubation with the repair enzymes, since the latter reduced the possibility of false negative results. The two adopted endpoints (comet and ENA assays) demonstrated complementary aptitudes, being recommended their jointly application since it allows a more effective genotoxic risk assessment.

Overall, the results obtained provided useful recommendations for policy-making, contributing to (re)formulate regulatory procedures for protecting the health of aquatic environment. In this direction, the data gathered in this work point to the importance of performing a genotoxic evaluation in order to actually determine the hazard posed by herbicides and their by-products. The magnitude of risk detected for both formulations strongly advise the adoption of restrictive measures in relation to their application in the proximity of watercourses. As mitigation measures, the development of formulations incorporating adjuvants selected on the basis of their lower toxicity emerged as a recommended path.

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# Chapter I

**General Introduction**

# 1 General Introduction

## 1.1 Environmental contamination and pesticides

The natural environment is being continuously loaded with foreign chemicals released by human activities such as industries and agriculture. Since the early sixties, especially due to the huge impact of the publication of Rachel Carson's book "Silent Spring" (Carson 1962), the world has become aware of the potential long-term adverse effects of pesticides in general, and their potential risks for the environment.

Within the environmental compartments, the aquatic has been widely studied and the concept "water quality" is considered as a vast and complex issue (Batista 2003). The aquatic environment covers two-thirds of the planet. Thus, the impacts on large aquatic systems – upon which many people depend – may pose a considerable threat to current and future generations. Economic functions for navigation, agriculture and energy supply, ecological functions for sustaining ecosystems, and social functions in terms of safety and drinking water supplies are without exception of critical importance to modern society (Brugge and Rotmans 2007).

The European Water Framework Directive (WFD), adopted in 2000, changed water management in all member states of the European Union, considering aquatic ecology as the base of management decisions (Hering et al. 2010) in order to protect aquatic environment in its entirety. In line with this, the WFD recognizes, for the first time, the integral nature of aquatic biota in determining the quality of European fresh and marine waters (Sweeting 2001) and considers as its main goal to achieve at least "Good Ecological Status" for all surface waters by 2015. Hence, the protection, improvement and sustainable use of Europe's water resources are a major goal of current European Water Policy.

Bearing this in mind, the WFD established the following objectives for water management in Europe:

- to prevent further deterioration of the water resources and enhance their status;
- to promote sustainable water use;
- to progressively reduce discharges of priority substances and to phase-out discharges of priority hazardous substances;
- to progressively reduce groundwater pollution;
- to contribute to mitigating the effects of floods and droughts (Mostert 2003).

The real challenge posed by the WFD for ecologists alike will be to integrate the scientific studies on separate habitat components and single taxonomic groups into a holistic view of the ecological status of aquatic compartments (Logan and Furse 2002).

Agricultural activities have been identified as major contributors to environmental contamination (Cooper 1993). The intensive use of pesticides in agriculture and forest plantations, that are usually sprayed during the cropping seasons (Candela 2003), represents a permanent risk of soil and water contamination due to their involvement in diverse environmental processes (Palma et al. 2004). As a consequence, the reference to these compounds whose concentration is above its maximum allowable value has also increased (PSEA 1997).

A pesticide is defined as any substance or mixture of substances intended for preventing, destroying, repelling or mitigating any pest (U.S.EPA 2012). An ideal pesticide should only affect target species and then be degradated into non-toxic substances, becoming thus safe to non-target organisms as well as environment friendly. The classification of pesticides can meet several criteria such as the target of action (e.g. insects, mites, nematodes, weeds, rats), the chemical class (e.g organochlorides, organophophates, carbamates), toxicological characteristics, mode of action as well as its origin (natural or synthetic) (Fernando and Duarte 2011). In Europe, and considering the pesticides categorization according to the specific type of pest that it is supposed to attack, the major types of pesticides in the market are fungicides (43%), followed by herbicides (36%), insecticides (12%) and other kind of pesticides (9%) (CEC 2002).

In general, after their application, pesticides reveal a complex environmental behaviour which results from several processes (physical, chemical and biological), influencing thus their transport and transformation. The knowledge associated to the mentioned processes assumes a great importance in what concerns to the improvement of the good practices in pesticides use.

The application of pesticides has become essential to control pests in modern agriculture, contributing significantly to enhance its productivity (Tomita and Beyruth 2002). Globally, 4.6 million tons of pesticides are annually sprayed into the environment. However, only 1% of that amount is effective, while 99% of pesticides applied reach non-target soils, water bodies and atmosphere, and finally being absorbed by non-target organisms (Zhang et al. 2011).

### 1.2 Herbicides in the aquatic environment

The important role of herbicides in the high-yield crops is well-established, since they are regular and intensively used during the production season as a way to control a variety of weeds in different crop types that compete with crop plants for water and

nutrients. Moreover, herbicides application in forest replantation areas increases the likelihood of success (Colborn and Short 1999).

In the 2000's, herbicides represented an huge amount of the pesticides used in Europe, where the highest quantities were usually applied in cereal crops (EC 2007; OECD 2008). In the particular case of Portugal, 2,042,383 kg of herbicide active ingredients were sold in 2010, reflecting an increase of 20%, when compared to 2009 data (DGADR 2011).

### 1.2.1 Fate and risk

Herbicides fate following the application can vary according to the nature and properties of the active substance, considering also the prevailing agro climatic conditions. The major input of herbicides in water systems occurs in an indirect way, during or shortly after application. Their diffuse losses are mostly constrained by their degradation, either abiotic (hydrolysis, oxidation/reduction) or due to the presence of microorganisms and dissipation processes, taking place in the topsoil layer. The herbicide and/or its metabolites can reach the water via artificial drainage systems, surface or sub-surface runoff, and leaching to groundwater. Additionally, the spray drift also appears as key potential route to surface water (Fig.1) (Batista 2003; Reichenberger et al. 2007; Marques 2009).

Despite the lower toxicity attributed to herbicides when compared to other pesticides, the concentrations required to an effective herbicidal action are high, posing thus a real risk to the aquatic environment (Sabater and Carrasco 1998; Sánchez et al. 2004; Marques 2009). Several authors demonstrated the effective risk of herbicides, namely to fish. Herbicides as clomazone, propanil, metsulfuron methyl, thiobencarb and 2,4-D showed to affect the fish acetylcholinesterase enzyme (AchE) (Sancho et al. 2000; Moraes et al. 2007; da Fonseca et al. 2008). Moreover, the latter and the organochlorides alachlor and paraquat showed to promote alterations in the antioxidant responses (Zhang et al. 2004; Parvez and Raisuddin 2006; da Fonseca et al. 2008) of fish. In addition, clomazone was also able to induce histopathological changes in silver catfish (Crestani et al. 2007).

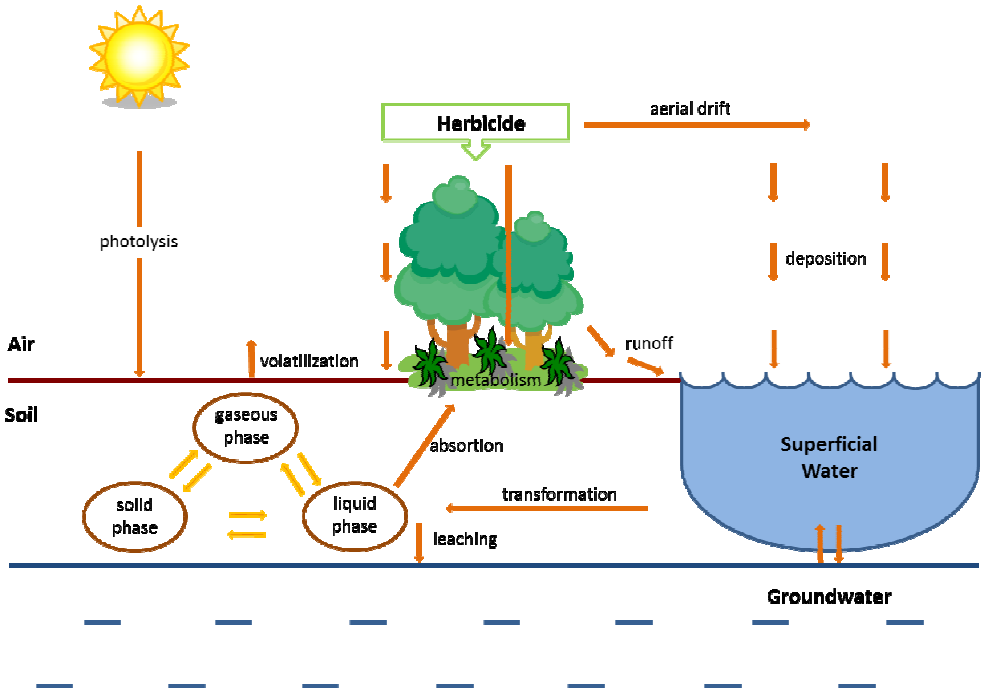


**Fig. 1.** Herbicides environmental behaviour (adapted from Batista 2003).

### 1.2.2 The cases of Roundup® and Garlon®

Glyphosate-based products are considered the most popular herbicides worldwide, due to its efficacy and cost effective (Monheit 2007). The commercial formulation Roundup® (distributed by Bayer CropScience) is a broad-spectrum organophosphate herbicide used to control undesirable weeds (Giesy et al. 2000) either in agriculture or ornamental gardens. It contains isopropylammonium salt of glyphosate at 485 $g.L^{-1}$, as active ingredient (equivalent to 360 $g.L^{-1}$ or 30.8 % of glyphosate), and polyethoxylated amine (POEA) (16 %), a non-ionic surfactant that promotes the penetration of glyphosate into the plant cuticle (Relyea 2005; Brausch and Smith 2007).

After the application, glyphosate (Fig. 2) is assimilated by leaves and other green plant structures, being then translocated within the phloem throughout the entire plant. Its mode of action consists primarily in the inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), a chloroplast-localized enzyme in the shikimic acid pathway of plants (Della-Cioppa et al. 1986) that prevents the production of the essential amino acids tryptophan, tyrosine, and phenylalanine, reducing thus the production of protein within the plant, thereby inhibiting plant growth (Herbicide Handbook 1994; Williams et al. 2000). Since this biochemical pathway does not exist in animals, glyphosate was supposed to be practically non-toxic to animal species (Monheit 2007).

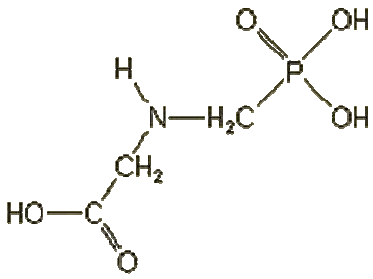


**Fig. 2.** Chemical structure of glyphosate (Source: FOOTPRINT PPDB).

The toxicity of the herbicide Roundup® was already demonstrated. Its ability in affecting antioxidant responses and acute physiological stress parameters (Langiano and Martinez 2008), as well as to promote the appearance of oxidative stress (Glusczak et al. 2007; Lushchak et al. 2009), is well documented.

The primary process of decomposition of glyphosate in the environment happens in the soil, due to the microbial degradation. This degradation generates aminomethylphosphonic acid (AMPA) (Fig. 3), the major metabolite of glyphosate (Forlani et al. 1999; Williams et al. 2000; Monheit 2007). This degradation may also occurs in the aquatic environment, where glyphosate can be naturally converted into sarcosine and aminomethylphosphonic acid (AMPA) (Landry et al. 2005; Al-Rajab et al. 2008), with a greater incidence of the latter. According to Giesy et al. (2000), the degradation of glyphosate in water bodies is relatively rapid, which may minimize its risk to the environment. On the other hand, the absence of studies concerning AMPA effects emphasizes the unpredictable risk to aquatic organisms.

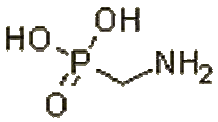


**Fig. 3.** Chemical structure of AMPA (Source: FOOTPRINT PPDB).

Garlon® is a commercial formulation (distributed by Dow AgroSciences) which presents triclopyr as its active ingredient (formulated as a butoxyethyl ester - TBEE - at a concentration equivalent to 480 $g.L^{-1}$) and kerosene (a petroleum distillate) as adjuvant. This herbicide is widely used for the control of woody plants and annual and perennial broadleaf weeds. Triclopyr (3,5,6-trichloro-2-pyridinyloxyacetic acid) (Fig. 4), belonging to the class of pyridinecarboxylic acids, acts as a selective systemic herbicide, mimicking the plant growth hormone auxin (indole acetic acid) and increasing its natural levels in 1000 times, causing the disruption of the hormonal balance and a subsequent uncontrolled and disorganized plant growth (DowElanco, 1996; Tu et al. 2001).

Once in the soil, TBEE is rapidly converted in triclopyr acid through microbial intervention (Tu et al. 2001), as well as by hydrolysis and photolysis (Health Canada 1991; Tu et al. 2001). Since the TBEE form is considered much more toxic than the acid (Kreutzweiser et al. 1995), its fast transformation in a less toxic form appears to be positive to the environment, at the time of the herbicide application (Health Canada 1991).

In what concerns to triclopyr persistence and mobility in the soil, and consequently the surface runoff, there are no consensus. However, its presence and potential toxic effects in aquatic organisms should not be neglected.

In any case, the commercial formulations containing the TBEE appear to be highly toxic to fish (Kreutzweiser et al. 1994; Kreutzweiser et al. 1995) and amphibians (Wojtaszek et al. 2005). In this context, the exposure to Garlon® pointed an increased risk of acute lethal effects on fish (Kreutzweiser et al. 1994), while Kreutzweiser and co-workers (1995) reported a growth inhibition in rainbow trout.

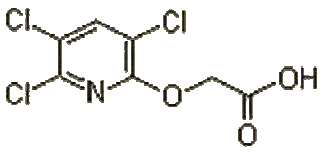


**Fig. 4.** Chemical structure of triclopyr (Source: FOOTPRINT PPDB).

## 1.3 DNA as a target molecule of herbicides

The DNA integrity is crucial to the organisms' survival. A single alteration in the DNA molecule could originate serious biological consequences, disrupting normal cell processes and leading to the cell death. The impact of genotoxic chemicals on the DNA integrity is considered one of the first events that occurs in exposed organisms (Frenzilli et al. 2009), highlighting thus the importance of its early evaluation. Since herbicides are considered as potential exogenous sources of DNA damage, there is a need to clarify the risks associated to these contaminants.

### 1.3.1 Genetic damage events

The environmental exposure to genotoxicants (substances capable to interfere with the DNA molecule) may be on the base of several DNA lesions.

DNA adducts are an example of a direct attack caused by genotoxic compound, for instance an herbicide. Adducts are products of the covalent reaction of electrophilic molecules with DNA. They represent an earlier event between the exposure to a genotoxic risk factor and a cancer. Their presence does not point automatically to an evolution towards the disease since they can be removed by DNA repair mechanisms. The appearance of these adducts may occur in different kinds of cells, depending on their localization, on the toxicokinetics, metabolism, DNA repair ability and cell proliferation rate of the tissue (De Flora et al. 1996). For instance, Peluso and coworkers found that the herbicide Roundup® was able to induce DNA adducts in mice (Peluso et al. 1998), while alachlor and its metabolite promote their appearance in bovines (Nelson and Ross 1998).

A genotoxic agent can also induce the formation of micronuclei (MN). Micronuclei are originated from chromosome fragment or whole chromosome that lag behind at anaphase, during the nuclear division (Fenech 2000). Micronuclei harboring chromosomal fragments may result from direct double-strand DNA breakage, conversion of single-strand breaks in double-strand breaks after cell replication, or inhibition of DNA synthesis (Mateuca et al. 2006). Micronuclei containing whole chromosomes are formed from defects in the chromosome segregation machinery such as deficiencies in the cell cycle controlling genes, failure of the mitotic spindle, kinetochore, or other parts of the mitotic apparatus or by damage to chromosomal substructures, mechanical disruption (Albertini et al. 2000) and hypomethylation of centromeric DNA (Fenech et al. 2005). Conventionally, it is assumed that MN are formed exclusively in dividing cells. However, it has been suggested that MN may also be originated through nuclear budding in interphase (Longwell and Yerganian 1965; Lindberg et al. 2007). Nuclear buds are

constituted by nuclear material which is associated to the main nucleus. It contains amplified DNA that is selectively localized to specific sites at the periphery of the nucleus and eliminated via nuclear budding to form MN during the S-phase of mitosis. Amplified DNA might be eliminated through recombination between homologous regions within amplified sequences forming minicircles of acentric and atelomeric DNA, which are localized in distinct regions within the nucleus or through the excision of amplified sequences after segregation to distinct regions of the nucleus. These facts suggest that the nucleus could have a capacity to detect excess DNA that does not fit well within the nuclear matrix, indicating a higher order DNA repair or nuclear housekeeping processes. Shimizu et al. (1998; 2000) have suggested that the nucleus may eliminate the excess of amplified DNA concentrating it in a peripheral point in the nucleus, following which this surplus DNA is budded out to form a micronucleus and eventually excluded from the cell altogether by extrusion of the micronucleus from the cytoplasm leading to the formation of a “minicell”. The process of nuclear budding occurs during S-phase and the nuclear buds are characterized by having the same morphology as a micronucleus with the exception that they are linked to the nucleus by a narrow or wide stalk of nucleoplasmic material depending on the stage of the budding process (Fenech 2002). These facts may shed a light in the controversial matter concerning the MN as an extreme stage of a nuclear abnormality (NA). Several studies have used the MN test to evaluate, in mice, the genotoxic potential of herbicides such as glyphosate and its commercial formulation Roundup® (Bolognesi et al. 1997), as well as atrazine and trifluralin (Gebel et al. 1997).

Moreover, the genotoxic damage is also evaluated analysing the occurrence of chromosomal aberrations (CA) and sister chromatid exchanges (SCE). Chromosomal aberrations are changes in normal chromosome structure or number that can occur spontaneously or as a result of a genotoxicant attack (Russell 2002). Structural CA may be induced by direct DNA breakage, by replication on a damaged DNA template, by inhibition of DNA synthesis and by other mechanisms (e.g. topoisomerase II inhibitors) (Albertini et al. 2000; Mateuca et al. 2006). Georgian and co-workers (1983) demonstrated the ability of the herbicide alachlor in induce CA in mice.

Sister chromatid exchanges occur during cell replication when a chromosome duplicates its genetic material, forming a pair of chromatids attached at the centromere. Through mechanisms that involve DNA breakage and rejoining, sister chromatids can exchange seemingly identical segments of DNA without known alterations of cell viability or function (Wilcosky and Raynard 1990). There are two models concerning the SCE formation. The recombination model is based on chromatid exchanges as part of a

postreplication repair process, whereas the replication model involves recombination during the DNA replication (Wilcosky and Raynard 1990). A study in humans showed that Roundup® was able to induce the formation of SCE (Vigfusson and Vyse 1980).

The association of herbicides genotoxicity with oxidative stress is not well established. The continuous production and subsequent release of reactive oxygen species (ROS) by the mitochondrial respiratory chain as well as their interference with cellular components and/or processes may result in oxidative stress. There are many different forms of ROS: singlet oxygen ($O_2$•), hydrogen peroxide ($H_2O_2$), superoxide ($O_2$•$^-$) and the hydroxyl radical (OH•). Depending on ROS levels, cellular death may occur. However, in normal conditions, the antioxidant defences are able to neutralize them, avoiding thus deleterious effects caused by either ROS higher levels production or due to the depletion of the antioxidant system (Collins 2009). To cope with oxidative stress and to prevent damaging effects of ROS, organisms increase their levels of protective antioxidants. In this context, thiols play an important role against the pernicious effects of pro-oxidant challenges where glutathione provides a first line of defence against ROS (Li et al. 2007; Oliveira et al. 2008). This non-enzymatic antioxidant is included in many cellular reactions, since it scavenges ROS directly and indirectly, through enzymatic reactions (Fang et al. 2002). Consequently, reduced glutathione (GSH) is converted to oxidized glutathione (GSSG), which is rapidly reduced back to GSH by glutathione reductase (GR), forming a closed system (redox cycle). The organic hydroperoxides reduction by GSH may be then catalyzed by glutathione peroxidase (GPx), which is provided by glutathione S-transferases (GST) (Wang and Ballatori 1998). Additionally, catalase (CAT), though not using GSH, plays also an important role in cellular protection reducing $H_2O_2$ to water and oxygen (Bainy et al. 1996). All these antioxidants exist as a coordinated system to detoxify distinct ROS. However, when antioxidant defences are impaired or surmounted it eventually may originate deleterious effects on cells and tissues such as DNA damage, enzymatic inactivation and peroxidation of cell constituents (Winston and Di Giulio 1991).

DNA is a key cellular component that is particularly susceptible to oxidative damage by ROS (Cerutti 1985). The DNA nature provides a useful substrate facilitating the formation of OH• adjacent to this critical biological target (Halliwell and Aruoma 1991) and allowing attacks to the sugar–phosphate backbone (Buxton et al. 1988; Valavanidis et al. 2006). In addition, the radical OH• may also induce a direct damage on the DNA, in particular guanine (Shugart 2000).

The pro-oxidant potential of herbicides has already been demonstrated (Banerjee et al. 2001). In particular, Lushchak and co-workers (2009) showed the herbicide Roundup® was able to induce mild oxidative stress in gold fish tissues. Other study pointed Roundup® as the responsible for the induction of the antioxidant enzyme catalase in fish (Langiano and Martinez 2008).

In what concerns to the herbicide Garlon®, as well as its active ingredient triclopyr, no studies were found concerning their pro-oxidant potential.

### 1.3.2 DNA damage tissue-specificity

It is already known that DNA strand breakage can present a tissue specificity (Siu et al. 2004; Pandey et al. 2006). In this direction, blood seems the preferred tissue to perform genotoxic studies, including in fish, mainly due to the easy sampling and adaptability of its cells to the most common methodologies, as well as the practical advantages of processing tissues constituted by nucleated and dissociated cells. However, circulating cells are frequently reported as the less sensitive, when compared to other types of cells (Frenzilli et al. 2009). Once in the blood, the genotoxicant is rapidly transported and distributed by the different organs, being thus accumulated according to its affinity to specific tissues. Considering the different roles performed by each tissue, it can be inferred that different cell types may respond to the presence of a genotoxicant with distinct sensitivities. Accordingly, 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 (Sharma et al. 2007).

Gills are the primary site of contact with waterborne genotoxicants, since they are in direct and continuous contact with the external medium, reflecting the uptake (Jiraungkoorskul et al. 2003). On the other hand, liver is considered as the central organ of metabolism in fish, since its role concerning bioaccumulation and biotransformation is stared of great important for activation and inactivation/detoxification of contaminants absorbed via different routes. Several studies reported liver (Liu et al. 2006; Mañas et al. 2009) and gills (Sharma et al. 2007; Cavalcante et al. 2008) as suitable tissues in the evaluation of genotoxicity.

Despite the integration of information, considering the system ‘‘organism–tissue–genotoxicant” to evaluate the genotoxicity (Kim and Hyun 2006) it is important to keep in mind that either the genotoxicant uptake routes or the bioaccumulation mechanisms should be carefully considered (Sharma et al. 2007), as well as genotoxicant specific characteristics.

1.3.3 Methodologies for the evaluation of genetic damage (DNA vs. chromosomal damage)

The application of genotoxic endpoints which meant to assess DNA damage intends to investigate an eventual and causal relationship between the exposure to genotoxicants and effects in individuals. In addition, the analysis of DNA alterations is considered a highly suitable approach for the evaluation of exposure to low concentrations of genotoxicants (Scalon et al. 2010), namely certain herbicides (Frenzilli et al. 2009). During the last decades, it was noticed the huge development of responsive genotoxic biomarkers, namely considering aquatic organisms (Hayashi et al. 1998).

(i) The comet assay

One of the most applied methodologies in the assessment of the genetic damage is the alkaline version of the Single Cell Gel Electrophoresis assay (SCGE), which was first developed by Singh and co-workers (1988). The comet assay is a simple, rapid, versatile, sensitive and economic method to evaluate DNA damage (Nandhakumar et al. 2011). Moreover, it allows the early detection of the genotoxic damage at the cellular level, requiring only a small number of cells. This technique reveals a broad spectrum of recent lesions that are susceptible of being repaired, as well as DNA strand-breaks and alkali labile sites (Lee and Steinert 2003; Andrade et al. 2004; Speit and Schütz 2008). Briefly, cells are embedded in agarose, on a microscope slide. Afterwards, a lysis with a detergent and a high concentration of NaCl is performed, in order to obtain nucleotides (histone-depleted DNA). In order to shed light on the eventual oxidative cause in the observed damage, it is possible to include an extra-step in the protocol where bacterial repair endonucleases detect oxidised bases (Collins 2009). Therefore, the use of formamidopyrimidine DNA glycosylase (FPG) and Endonuclease III (EndoIII) increased the sensitivity of the assay, enhancing the detection of a wider range of damage, and essentially its specificity (Azqueta et al. 2009). Later, an electrophoresis is carried out. Comet-like structures are then stained and observed with a fluorescence microscope. The DNA damage levels (based on a visual scoring) are inferred considering the tail length and intensity supported in five comet classes, from 0 (no tail) to 4 (almost all DNA in tail) (Collins 2004; Azqueta et al. 2009). This assay has been widely used to evaluate the genotoxic potential of herbicides in fish (Bolognesi et al. 1997; Clements et al. 1997; Moretti et al. 2002; Çavas and Könen 2007; Cavalcante et al. 2008).

(ii) Erythrocytic Nuclear Abnormalities (ENA) assay

The ENA assay consists in the detection of micronuclei and other nuclear abnormalities in nucleated mature erythrocytes (Pacheco and Santos 1997). Nuclear abnormalities were first described by Carrasco et al. (1990). The determination of the nuclear abnormalities frequency is based on the following nuclear lesion categories: kidney shaped nuclei (K), lobed nuclei (L), binucleate or segmented nuclei (S) and micronuclei (MN). These nuclear deformations are signals of chromosome breakage (clastogenicity) or loss and mitotic spindle apparatus dysfunction (aneugenicity) (Fenech 2000; Stoiber et al. 2004). Contrarily to what was stated for the lesions detected by the comet assay, these lesions are almost irreparable, being considered as less transient alterations, displaying a later appearance. ENA assay showed also to be a suitable marker for the evaluation of genotoxic damage of herbicides in fish (Çavas and Könen 2007).

## 1.4 Genotoxic risk of Roundup® and Garlon® in fish - an almost unexplored issue

As mentioned above, herbicides may be drifted to non-target areas, following applications, posing thus an eventual genotoxic risk to aquatic organisms.

The Unites States Environmental Protection Agency (USEPA) classified glyphosate as moderately toxic to practically non-toxic to fish (USEPA 1993). However, and despite the controversy concerning the ingredients of glyphosate-based herbicides, little is known about their genotoxic potential. Only a few studies, concerning the commercial formulation Roundup®, demonstrated its genotoxicity to fish expressed as cytogenetic and DNA damaging effects (Grisolia 2002; Çavas and Könen 2007; Cavalcante et al. 2008).

Considering the fact that glyphosate is rapidly converted into its breakdown product AMPA, and since the latter is reported as a recurrent aquatic environment pollutant, it would be expectable that its genotoxicity to aquatic organisms have been better studied. Despite this, no studies were performed considering these organisms. In fact, and in what concerns to the genotoxic potential of AMPA, only a single study was performed, revealing its ability in inducing genotoxicity in human lymphocytes and mice (Mañas et al. 2009).

As observed, little is known about the pernicious effects of Garlon® and its related products, remaining their genotoxic potential unknown.

Therefore, regarding the widely use of these herbicides, and their subsequent entrance in the water bodies, it should be highlighted the extreme importance of evaluating their genotoxicity in aquatic organisms.

### 1.5 Goals and thesis structure

In the context of the herbicides genotoxicity to fish, the main goals of the present study were: (i) to assess the genotoxic potential of the formulations Roundup® and Garlon® and, elucidating the contribution of their constituents to the overall effect; (ii) to develop a biologically base knowledge on DNA damage mechanisms; (iii) to contribute to mitigating the effects of agrochemicals in aquatic biota, namely fish, providing scientific data able to improve forestry and agriculture managing practices.

In order to achieve these general goals, the following specific objectives were defined:

- to differentiate the type of damage (DNA and chromosomal damage) induced after exposures to environmental realistic concentrations;
- to elucidate the tissue-specificity of the responses to the genotoxic stimuli;
- to clarify the involvement of defence mechanisms in the DNA damage extent;
- to identify the involvement of oxidative damage on the DNA integrity loss, namely through the use of DNA lesion-specific repair enzymes (FPG and EndoIII).

This study was carried out through the implementation of short-term (1 to 3 days) laboratory experiments, using the European eel (*Anguilla anguilla* L.) as test organism, selected due to its previous successful adoption in genotoxicity evaluation.

The present thesis comprises eight chapters. The first chapter (I) is of introductory nature, addressing various aspects important for understanding the global perspective of this thesis. Chapters II to VII are concurrent with the above mentioned specific objectives, and the final chapter (VIII) consists of a general discussion, where all of the results obtained and reported in the previous chapters are discussed, providing a global and integrated perspective.

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# Chapter II

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

*Mutagenesis* 25(5), 523–530 (2010)

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

**Abstract**

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 $\mu g.L^{-1}$), addressing also the possible association with oxidative stress. Thus, comet and erythrocytic nuclear abnormalities (ENA) assays were adopted, as genotoxic endpoints, reflecting different types of genetic damage. The pro-oxidant state was assessed through enzymatic (catalase, glutathione-S-transferase, glutathione peroxidase, 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 days exposure to both tested concentrations. In addition, the two genotoxic indicators were positively correlated. Antioxidant defenses 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.

**Keywords**: Roundup®; glyphosate; genotoxicity; oxidative stress; fish.

## 2.1 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, since they easily reach the water bodies, mainly through soil surface run-off (Tomita and Beyruth 2002). Compounds based on glyphosate are broad-spectrum nonselective organophosphate herbicides and the most extensively used worldwide (Cavalcante et al. 2008). The herbicide Roundup® is a commercial formulation which contains glyphosate (N-(phosphonomethyl) glycine) as the active ingredient and polyethoxylene amine (POEA) as surfactant. In natural water bodies, Roundup® (measured as glyphosate acid equivalents) has been detected at concentrations from 0.01 to 0.7 $mg.L^{-1}$ (Paveglio et al. 1996; Peruzzo et al. 2008), reaching 1.7 $mg.L^{-1}$ in an extreme situation after direct application to water (Horner 1990). Despite the acute toxicity of glyphosate was considered to be low (Li and Long 1988; WHO 1994), glyphosate-based commercial formulations are generally more toxic than pure glyphosate (Rank et al. 1993; Peixoto 2005) mainly due to the interference of surfactants such as POEA (Tsui and Chu 2008).

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 (Romeo and Quijano 2000; Sharma et al. 2007). Moreover, fish showed to be more sensitive to Roundup® than terrestrial organisms (Giesy et al. 2000; Grisolia 2002) highlighting the ecotoxicological relevance to approach this taxon. Despite the description of a variety of deleterious effects induced by Roundup® in fish (Jiraungkoorskul et al. 2003; Glusczak et al. 2006; Glusczak et al. 2007; Lushchak et al. 2009), 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 (Grisolia 2002; Çavas and Könen 2007; Cavalcante et al. 2008). 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 (Muniz et al. 2008). In addition to damaging DNA, ROS also disrupt the function of DNA repair proteins (Shimura-Miura et al. 1999). To counteract ROS-induced damage, cells evolved

antioxidant systems as a major defense mechanism. Thus, variations in the antioxidant defenses can be very sensitive in revealing a pro-oxidant condition and have been proposed as indicators of pollutant mediated oxidative stress (Ahmad et al. 2006; Oliveira et al. 2008). Human and mammal studies with pesticides, especially organophosphates, demonstrated that DNA damage and oxidative stress are mechanistically linked (Lodovici et al. 1997; Muniz et al. 2008). Though Roundup® induced mild oxidative stress in goldfish (Lushchak et al. 2009), 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 $\mu g.L^{-1}$), addressing its possible association with oxidative stress. Genotoxic endpoints such as comet and erythrocytic nuclear abnormalities (ENA) 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 (Lee and Steinert 2003; Andrade et al. 2004), 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 (Pacheco and Santos 1997), signals in vivo chromosome breakage (clastogenicity) or loss and mitotic spindle apparatus dysfunction (aneugenicity) (Fenech 2000; Stoiber et al. 2004). 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.

## 2.2 Material and Methods

### 2.2.1 Chemicals

A commercial formulation of glyphosate (N-(phosphonomethyl) glycine) - Roundup® - 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 POEA (16 %) as surfactant, was used. All the other chemicals were obtained from the Sigma–Aldrich Chemical Company (Spain).

### 2.2.2 Test animals and experimental design

European eel (*Anguilla 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 physico-chemical conditions: salinity 0, temperature 20±1 ºC, pH 7.3±0.2, ammonia <0.1 $mg.L^{-1}$, dissolved oxygen 8.1±0.5 $mg.L^{-1}$.

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 6 aquaria (6 fish per aquaria; 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 - 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 (approximately 400 µL) from the posterior cardinal vein using heparinised Pasteur pipettes. Blood smears were immediately prepared for ENA assay. Two µL of blood were diluted in 1 mL of PBS for comet assay. The remainder volume was stored in microtubes and kept at -80 ºC until further procedures for oxidative stress analyses. Following sampling, fish were sacrificed by cervical transaction.

### 2.2.3 Evaluation of genetic damage

#### 2.2.3.1 Comet assay

The alkaline version of the comet assay was performed according to Collins (2004) methodology with slight modifications. Two gel replicates, containing each one approximately $2x10^4$ cells (using the whole blood previously diluted in PBS) in 70 µL of 1% low melting point agarose in PBS, were placed on one glass microscope slide, precoated with 1% normal melting point agarose. The gels were covered with glass cover slips, left for ±5 minutes at 4 ºC 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 ºC, for 1 h. Slides were immediately processed according to the conventional comet assay (Collins 2004). 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 $V.cm^{-1}$ (achieved by adjusting the total volume of buffer). The slides were stained with ethidium bromide (20 $\mu g.mL^{-1}$). One slide with two gels (100 nucleoids per gel) was observed for each fish using a Leica DMLS fluorescence microscope (400x 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:

GDI = [(% nucleoids class 0)×0] + [(% nucleoids class 1)x1] + [(% nucleoids class 2)×2] + [(% nucleoids class 3)×3] + [(% nucleoids class 4)×4]

Results were expressed as "arbitrary units" in a scale of 0 to 400 per 100 scored nucleoids (as average value for the 2 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. (2009).

#### 2.2.3.2 ENA assay

This assay was carried out in mature peripheral erythrocytes according to the procedure of Pacheco and Santos (1996). 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 1000x 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 (2000) and Ayllon and Garcia-Vazquez (2001). Final results were expressed as the mean value (‰) of the sum for all the lesions observed (K+L+S+N+MN).

### 2.2.4 Biochemical analyses

#### 2.2.4.1 Tissue preparation and fractionation

Whole-blood samples were lysed through homogenization in a 1:15 ratio (blood volume:buffer volume), using a Potter-Elvehjem homogenizer, 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 ºC. Aliquots of PMS were stored in microtubes at -80 ºC until analyses.

2.2.4.2 Measurement of antioxidant responses and peroxidative damage

CAT activity was assayed (at 25 ºC) by the method of Claiborne (1985) as described by Giri et al. (1996). 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 $protein^{-1}$ ($\varepsilon$=43.5 $M^{-1}.cm^{-1}$).

GST activity was determined (at 25 ºC) using CDNB (1-chloro-2,4-dinitrobenzene) as a substrate, according to the method of Habig et al. (1974). Absorbance was recorded spectrophotometrically at 340 nm for 3 min. The enzyme activity was calculated as nmol CDNB conjugate formed $min^{-1}$.mg $protein^{-1}$ ($\varepsilon$=9.6 $mM^{-1}.cm^{-1}$).

GPx activity was determined (at 25 ºC) according to the method of Mohandas et al. (1984). NADPH oxidation was recorded spectrophotometrically at 340 nm and GPx activity was calculated in terms of nmol NADPH oxidized $min^{-1}$.mg $protein^{-1}$ ($\varepsilon$=6.22 x 103 $M^{-1}.cm^{-1}$).

GR activity was assayed (at 25 ºC) by the method of Cribb et al. (1989). 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 nmol of NADPH oxidised $min^{-1}$.mg $protein^{-1}$ ($\varepsilon$=6.22×103 $M^{-1}.cm^{-1}$).

For GSHt quantification, protein content in the tissue lysate was precipitated with trichloro acetic acid (12 %) for 1 h and then centrifuged at 13,400 g for 20 min at 4 ºC. The resulting supernatant was collected and stored at –80 ºC. GSHt was determined (in deproteinated PMS, at 25 ºC) 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 colored 5-thio-2-nitrobenzoic acid (TNB). The rate of TNB production is proportional to the concentration of glutathione in the sample (Tietze 1969). Formation of TNB was measured by spectrophotometry at 412 nm and the results expressed as nmol TNB formed $min^{-1}$.mg $protein^{-1}$ ($\varepsilon$=14.1 $mM^{-1}.cm^{-1}$).

As estimation of lipid peroxidation (LPO), TBARS quantification was carried out in the previously prepared lysate (treated with 1-1 butylated hydroxytoluene 4% in methanol to prevent oxidation) as adapted by Filho et al. (2001). The absorbance was measured at 535 nm and the rate of LPO was expressed in nmol of TBARS formed per mg of fresh tissue ($\varepsilon$=1.56x105 $M^{-1}.cm^{-1}$).

Total protein contents were determined according to the Biuret method (Gornall et al. 1949), using bovine serum albumin as a standard.

#### 2.2.5 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 as well as 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, the non parametric correspondent test (Kruskall Wallis) was performed, followed by the non parametric all pairwise multiple comparison procedure (Dunn's test) (Zar 1996). 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 (Zar 1996).

### 2.3 Results

#### 2.3.1 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 (Fig. 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 $\mu g.L^{-1}$ 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 days exposure as exposed groups displayed damage increments of 1.4 and 1.7 times, respectively for 58 and 116 $\mu g.L^{-1}$ Roundup®. The difference between exposed groups was less pronounced (1.2 times increase for 116 $\mu g.L^{-1}$) than that observed after 1 day exposure. Globally, the GDI results were concentration dependent, whereas no time-related alterations were noticeable.

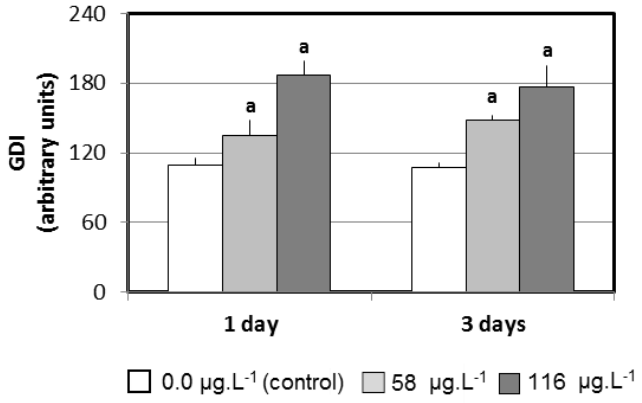


**Fig. 1.** Mean GDI (expressed as arbitrary units), measured by comet assay, in peripheral blood cells of *A. anguilla* exposed to 58 and 116 µg.L$^{-1}$ Roundup®, during 1 and 3 days. Letter (a) denotes statistically significant differences ($p<0.05$) *vs*. control. Bars represent the standard error.

Individual DNA damage classes (Table 1), unlike the pattern displayed in total DNA damage (GDI; Fig. 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$^{-1}$ Roundup® and control, whereas fish exposed to 58 µg.L$^{-1}$ Roundup® showed significant increases only in classes 1 and 2. Following 3-day exposure, classes 1, 2 and 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$^{-1}$ group.

**Table 1.** 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$^{-1}$ Roundup®, during 1 and 3 days. Statistically significant differences ($p<0.05$) are: (a) vs. control; (♦) vs. 1-day exposure (for the same exposure condition).

| Exposure Time | Roundup® Concentration (µg.L$^{-1}$) | Damage Classes | | | | |
|---|---|---|---|---|---|---|
| | | 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 |

2.3.2 ENA frequency

No significant alterations were found in ENA frequency following the first day of exposure (Fig. 2). However, an increased tendency was perceptible in both Roundup® concentrations, relatively to control.

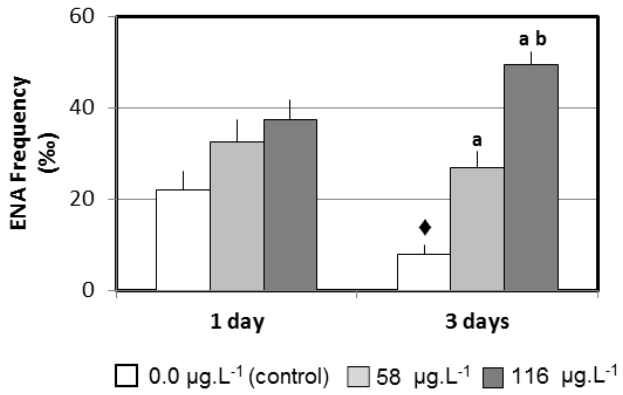


**Fig. 2.** Mean frequency (‰) of erythrocytic nuclear abnormalities (ENAs) in *A. anguilla* exposed to 58 and 116 µg.L$^{-1}$ Roundup®, during 1 and 3 days. Statistically significant differences ($p<0.05$) are: (a) vs. control and (b) vs. 58 µg.L$^{-1}$ (within the same exposure duration); (♦) vs. 1-day exposure (for the same exposure condition). Bars represent the standard error.

A similar pattern was observed when each lesion category was considered individually (Table 2). Nevertheless, significant ENA increases were found after 3 days exposure in both exposed groups in comparison with the control. In addition, a significant ENA increase (around 2 times) was observed from 58 to 116 µg.L$^{-1}$ 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$^{-1}$ Roundup® when compared to 58 µg.L$^{-1}$. Lobed nuclei was the most commonly detected abnormality in fish exposed for 3 days to 58 µg.L$^{-1}$ Roundup® (L>K>S>N=MN), whereas in 116 µg.L$^{-1}$ 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- up to 3-day exposure in control group in terms of L (Table 2) and total ENA (Fig. 2) frequencies, while in the 116 µg.L$^{-1}$ group a significant increase was observed in K frequency.

**Table 2.** Mean frequency (‰) of each nuclear abnormality category (± standard error) in peripheral erythrocytes 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).

| | | Nuclear Abnormality Categories | | | | | |
|---|---|---|---|---|---|---|---|
| Exposure Time | Roundup® Concentration (µg.L$^{-1}$) | Kidney shaped (K) | Lobed (L) | Segmented (S) | Notched (N) | Sub-total (K+L+S+N) | Micronuclei (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♦ | 0.40±0.40 | 0.00±0.00 | 8.00±1.82♦ | 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[ab]♦ | 20.60±1.08[ab] | 0.80±0.80 | 0.00±0.00 | 49.40±2.89[ab]♦ | 0.00±0.00 |

2.3.3 Antioxidant responses and lipid peroxidative damage

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

TBARS values after 1-day exposure to 116 µg.L$^{-1}$ Roundup®, when compared to control as well as to 58 µg.L$^{-1}$ 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$^{-1}$ groups.

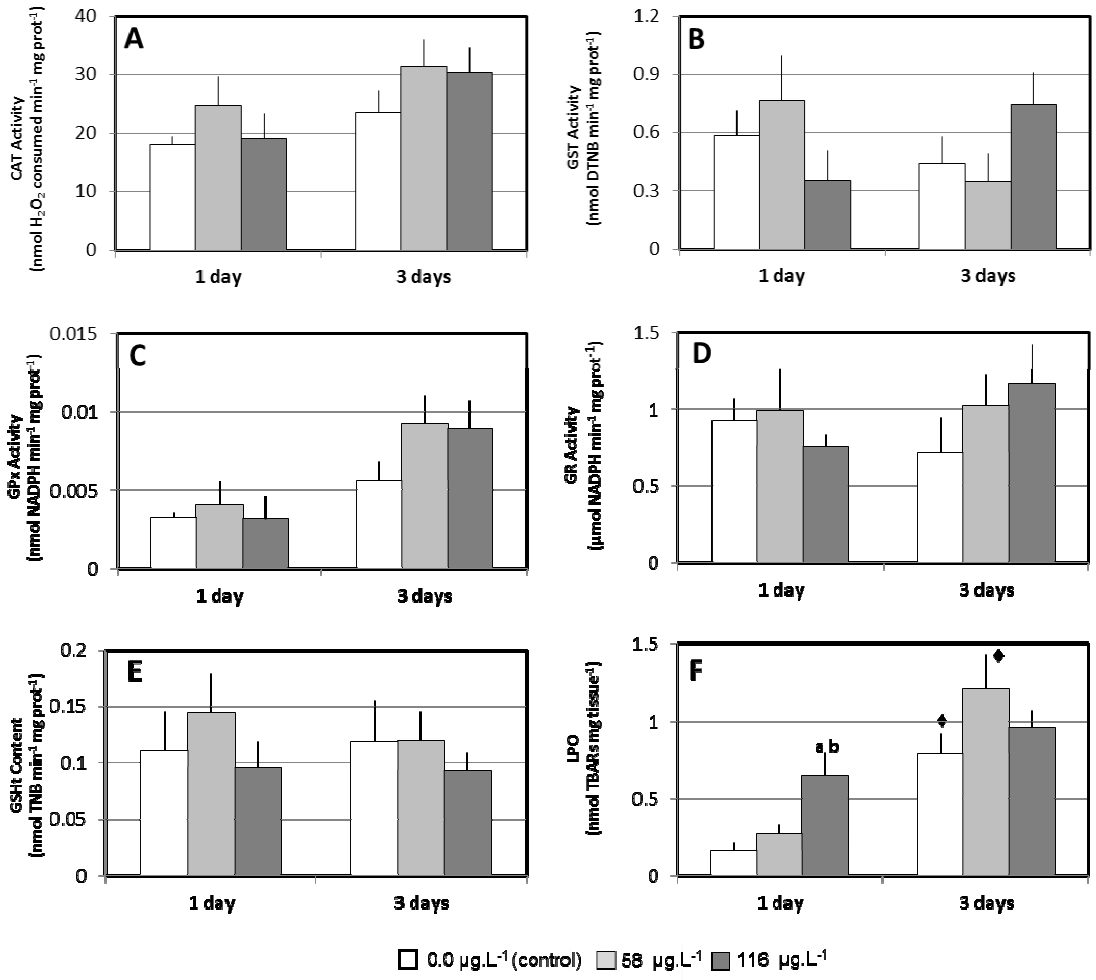


**Fig. 3.** Mean catalase (CAT) (A), glutathione-S-transferase (GST) (B), glutathione peroxidase (GPx) (C) and glutathione reductase (GR) (D) activities, as well as total glutathione (GSHt) content (E) and lipid peroxidation (LPO) levels (F) in peripheral blood of *A. anguilla* exposed to 58 and 116 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.

2.3.4 Correlations between biological parameters

The correlation between all biological parameters was statistically tested by analyzing the data obtained after 1- and 3-day exposures to 58 and 116 µg.L$^{-1}$ Roundup®. However, only the correlation between DNA damage and ENA frequency was statistically significant (Fig. 4).

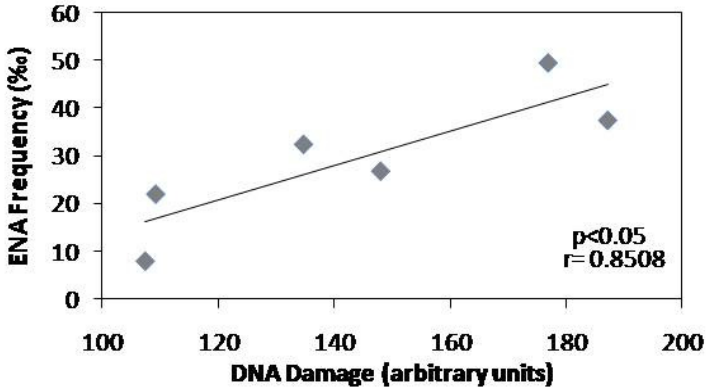


**Fig. 4.** Correlation between DNA damage (measured by comet assay) and ENA frequency in peripheral blood of *A. anguilla* exposed to 58 and 116 µg.L$^{-1}$ Roundup®, during 1 and 3 days. Statistical significance and correlation coefficient are represented by p and r, respectively.

## 2.4 Discussion

A model to estimate the worst-case exposure conditions was developed by Giesy et al. (2000) who set 0.271-0.406 and 0.339-0.677 mg.L$^{-1}$ 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 (Horner 1990). In this perspective, the Roundup® concentrations tested in the present study (58 and 116 µg.L$^{-1}$) are realistic, mainly in the context of agriculture applications (the primary use of Roundup®), representing an important progress in relation to previous fish studies (Çavas and Könen 2007; Cavalcante et al. 2008) 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 endpoints 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 (Pacheco and Santos 1996, 1998). 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 utilization. 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 (Ruas et al. 2008).

### 2.4.1 Genotoxic damage induced by Roundup®

Analyzing 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 analyzed individually. Hence, after 1-day exposure only 116 $\mu g.L^{-1}$ 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 $\mu g.L^{-1}$ 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 $\mu g.L^{-1}$, 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 Çavaş and Könen (2007) and Cavalcante et al. (2008) 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 $mg.L^{-1}$ for 1 day (without test water renewal) exhibited significantly elevated comet scores after 6 h exposure, returning to control levels 18 h later (Cavalcante et al. 2008). This pattern differs from the present study which shows elevated comet scores up to 3 days 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 cells catabolism by the spleen. These processes were previously presented by Saleha Banu et al. (2001) 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 mosambica*) exposed to an organophosphate pesticide. Moreover, an increased splenic erythrophagia was associated to intense genetic damage in *A. anguilla* (Pacheco and Santos 2002).

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 Çavaş and Könen (2007) with *Carassius auratus*, though the concentrations tested by this researchers were substantially higher (5, 10 and 15 $mg.L^{-1}$). 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 o 10 $mg.L^{-1}$ for 6, 24 and 96 h (Cavalcante et al. 2008).

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 (Udroiu 2006), 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. (2007), DNA damage measured by the comet assay appears earlier than do micronuclei 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 endpoints provide complementary information, allowing a more effective assessment of Roundup® genotoxic effects when jointly applied. Accordingly, Wirzinger et al. (2007) 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 (Russo et al. 2004) and negative (Wirzinger et al. 2007) correlations have already been observed in fish. The present results seem to support the hypothesis of Russo et al.

(2004) 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 trombocytes). 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.

### 2.4.2 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 (Glusczak et al. 2007; Langiano and Martinez 2008; Lushchak et al. 2009). The few available studies provided inconclusive information, due to the variety of species and concentration ranges adopted, as well as the target organs analyzed. Thus, Roundup® exposure (2.5-20 $mg.L^{-1}$) generally suppressed the activities of superoxide dismutase (SOD), GST and GR in brain, kidney and liver of *C. auratus* (Lushchak et al. 2009), which was explained by a ROS-induced inactivation. Oppositely, liver CAT activity increased in *C. auratus* (only at 10 $mg.L^{-1}$) (Lushchak et al. 2009) and *P. lineatus* (7.5-10 $mg.L^{-1}$) (Langiano and Martinez 2008), whereas in *Rhamdia quelen* (0.2-0.4 $mg.L^{-1}$) it remained unaltered (Glusczak et al. 2007).

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 defenses 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 (Lushchak et al. 2009) 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. (2009) 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. (2007) 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. (2006) who stated that LPO increase cannot be predicted only on the basis of antioxidant variations.

Elevated levels of ROS or depressed antioxidant defenses 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 (Collins 2004; Azqueta et al. 2009). This association has already been demonstrated in humans for organophosphate pesticides (Muniz et al. 2008). Though never assessed, oxidative stress was hypothesized as a possible mechanism for Roundup® genotoxic action in fish (Cavalcante et al. 2008). 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 recognize oxidized 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. (2001), 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 (Wild 1975), affecting DNA bases either directly or indirectly via protein alkylation (Mohan 1973; Green et al. 1974).

## 2.5 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 endpoints 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.

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**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.

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# Chapter III

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

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

**Abstract**

Organophosphate herbicides are among the most dangerous agrochemicals for 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 Roundup® genotoxic potential to the teleost *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 1 and 3 days. The standard procedure of comet assay was applied to gill and liver cells in order to reflect organ-specific genetic damage. Since liver is a central organ on 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 DNA damage increase for both Roundup® concentrations and exposure times, though 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 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 compulsory condition for the induction of DNA damaging effects of Roundup®. Differently, by increasing the exposure length (to 3-day), ROS-dependent processes gained preponderance as a mechanism of DNA damage in the higher concentration.

**Keywords**: Roundup®; glyphosate; genotoxicity; oxidative stress; fish.

### 3.1 Introduction

The increment in the use of pesticides in contemporary agriculture is considered a major problem worldwide. Though the application of these agrochemicals is concentrated in terrestrial areas, they can reach the aquatic environment by drift, runoff, drainage and leaching (Cerejeira et al. 2003), raising a number of environmental concerns especially in systems of shallow waters. Among pesticides, organophosphates constitute the predominant class (Kaur et al. 2007). In this context, the use of Roundup®, a glyphosate-based non-selective herbicide, has increased mainly due to the cultivation of genetically modified crops (Giesy et al. 2000). As a consequence of the extensive use of this commercial formulation, glyphosate has been widely detected in water bodies (Battaglin et al. 2005; Kolpin et al. 2006; Blanchoud et al. 2007; Pesce et al. 2008), increasing significantly the risks to non-target organisms, namely fish (Çavas and Könen 2007).

Though some studies have considered glyphosate only slightly toxic for aquatic animals (USEPA 1993; WHO 1994) and with low potential to bioaccumulate (WHO 1994), glyphosate-based formulations are generally more toxic than pure glyphosate (Rank et al. 1993; Peixoto 2005) mainly due to the interference of surfactants (Tsui and Chu 2008). Despite the existence of a large body of work concerning Roundup® deleterious effects on fish, only a few studies addressed its genotoxic potential. The available data demonstrated the genotoxicity of Roundup® to fish, expressed as cytogenetic and DNA-damaging effects (Grisolia 2002; Çavas and Könen 2007; Cavalcante et al. 2008). Nevertheless, the concentrations tested in these studies were excessively high, when compared to 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 (Guilherme et al. 2010).

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 well-known process underlying genotoxicity, namely in the context of environmental genotoxicants (Collins 2004; Azqueta et al. 2009). Since organophosphate pesticides are known as oxidative stress inducers (Banerjee et al. 2001), the hypothesis that DNA damage induced by Roundup® may have an oxidative cause should be considered. This association has already been demonstrated in humans for organophosphate pesticides (Muniz et al. 2008). In relation to fish, the only existent study demonstrated that DNA and chromosomal damage induced by Roundup® in blood cells

was not supported by an increased pro-oxidant state, evaluated by the antioxidant responses (Guilherme et al. 2010). This study also recommended the assessment of DNA bases oxidation (for instance, applying the comet assay with an extra step of digesting the nucleoids with enzymes that specifically recognise oxidised bases) as a more straightforward strategy to achieve the aimed mechanistic knowledge.

Genotoxic studies in fish are frequently performed in erythrocytes, due to their easy sampling and adaptability to most common methodologies (Ayllon and Garcia-Vazquez 2001; Grisolia and Starling 2001). However, according to Sharma et al. (2007), other cell types should be used for monitoring genotoxic effects, thereby exploiting tissue-specific responses and acquiring a better perspective of the overall condition of the organisms. When waterborne contamination is considered, gills are the first target organ due to the wide surface area in direct and continuous contact with the external medium and its involvement on uptake (Al-Sabti and Metcalfe 1995; Jiraungkoorskul et al. 2003). Additionally, liver is also of great interest in individual fish health assessment considering its multi-functionality and primary role in the metabolism of xenobiotics, essential for activation/inactivation and detoxification of contaminants absorbed from different routes (Cizdziel et al. 2003). Moreover, fish exposure to Roundup® induced histological injuries in both organs (Jiraungkoorskul et al. 2002), despite antioxidant alterations were only demonstrated in liver (Langiano and Martinez 2008; Lushchak et al. 2009).

Considering that genotoxicity stands for the most adverse impact of chemicals on wild organisms and the knowledge gaps previously recognized, 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 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, 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.

## **3.2** Material and Methods

### 3.2.1 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 needed to perform comet assay, as well as to quantify antioxidants, were obtained from the Sigma–Aldrich Chemical Company (Spain).

### 3.2.2 Test animals and experimental design

European eel (*Anguilla 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 physico-chemical conditions: salinity 0, temperature 20±1ºC, 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 above described for the acclimation period. Fish were not fed 1 day before the experiment is started or during experimental period. Thirty-six eels were divided into 6 aquaria (6 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 – negative control groups. For each pesticide concentration, 1- and 3-day exposures were tested, corresponding to the two different aquaria previously mentioned. No mortality was observed during the whole experiment. After each exposure time, 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 comet assay and the remainder tissue was stored in microtubes, frozen in liquid nitrogen and kept at -80 ºC until further procedures for antioxidants analyses.

### 3.2.3 Evaluation of genetic damage

#### 3.2.3.1 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 fine minced tissue pieces (Hartmann et al. 2003). The conventional alkaline version of the comet assay was performed according to the methodology of Collins (2004) with slight modifications. Two gel replicates, containing each one approximately $2x10^4$ cells (using cell suspension in PBS) in 70 µL of 1% low melting point agarose in PBS, were placed on one glass microscope slide, precoated with 1% normal melting point agarose. The gels were covered with glass coverslips and left for ±5 min at 4 ºC in order to solidify agarose, and then emerged in a lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 1% Triton X-100, pH 10) at 4 ºC, for 1 h. Then, slides were 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 $\mu g.mL^{-1}$).

Considering liver, an additional set of slides was prepared to apply the comet assay procedure 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 as well as other altered purines (ring-opened purines or formamidopyrimidines), into DNA single strand breaks (Azqueta et al. 2009). Thus, after lysis of agarose-embedded cells, slides were washed 3 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 ºC. Then, 50 µL of FPG in buffer was applied in the centre of each gel, along with a coverslip, prior to incubation at 37 ºC for 45 min in a humidified atmosphere. Another set of slides was submitted to the same treatment, though 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, was observed for each fish and organ, using a Leica DMLS fluorescence microscope (400x 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:

GDI = [(% nucleoids class 0)×0] + [(% nucleoids class 1)x1] + [(% nucleoids class 2)×2] + [(% nucleoids class 3)×3] + [(% nucleoids class 4)×4]

GDI results were expressed as arbitrary units in a scale of 0 to 400 per 100 scored nucleoids (as average value for the 2 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. (2009). In order to improve the expression of the DNA damage extent, the sub-total frequency of nucleoids with medium (class 2), high (class 3) and complete (class 4) damaged DNA was also calculated (Palus et al. 1999; Çavas and Könen 2007).

As positive control, both gill and liver cells were treated with 50 µM hydrogen peroxide (Sigma-Aldrich, Spain) for 5 min, according to Collins et al. (1995), and the respective GDI values were scored.

### 3.2.4 Antioxidant system analyses

#### 3.2.4.1 Tissue preparation and fractionation

Both organs (gills and liver) were homogenized in a 1:10 ratio (tissue volume: buffer volume), using 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 ºC. Aliquots of PMS were stored in microtubes at -80 ºC until analyses.

#### 3.2.4.2 Measurement of antioxidant responses

Superoxide dismutase was assayed (at 25 ºC) using a Ransod kit (Randox Laboratories Ltd., UK). Briefly, 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 that which 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 ºC) by the method of Claiborne (1985) as described by Giri et al. (1996). 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 protein$^{-1}$ ($\varepsilon$ = 43.5 M$^{-1}$.cm$^{-1}$).

Glutathione-S-transferase activity was determined using CDNB (1-chloro-2,4-dinitrobenzene) as a substrate, according to the method Habig et al. (1974). The assay was carried out at 25 ºC 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 protein$^{-1}$ ($\varepsilon$ = 9.6 mM$^{-1}$.cm$^{-1}$).

Glutathione peroxidase activity was determined (at 25 ºC) according to the method of Mohandas et al. (1984), 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 oxidized.min$^{-1}$.mg protein$^{-1}$ ($\varepsilon$ = 6.22 x 10$^3$ M$^{-1}$.cm$^{-1}$).

Glutathione reductase activity was assayed (at 25 ºC) by the method of Cribb et al. (1989), with some modifications. The assay determines indirectly the GR activity by measuring the NADPH disappearance associated to the 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 (1mM) 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 protein$^{-1}$ ($\varepsilon$ = 6.22×10$^3$ M$^{-1}$ cm$^{-1}$).

For GSHt quantification, protein content in the tissue lysate was precipitated with trichloro acetic acid (TCA 12 %) for 1 h and then centrifuged at 13,400 g for 20 min at 4 ºC. The resulting supernatant was collected and stored at –80 ºC. GSHt was determined (in deproteinated PMS, at 25 ºC) 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 colored 5-thio-2-nitrobenzoic acid (TNB). The rate of TNB production is directly proportional to the concentration of glutathione in the sample (Tietze 1969; Baker et al. 1990). 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 gluthatione (GSHt) content. The results were expressed as nmol TNB formed.$min^{-1}$.mg $protein^{-1}$ ($\varepsilon$ = 14.1 $mM^{-1}.cm^{-1}$).

Total protein contents were determined according to the Biuret method (Gornall et al. 1949), using bovine serum albumin (Merck) as a standard.

### 3.2.5 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 as well as 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 correspondent test (Kruskall Wallis) was performed, followed by a non parametric all pairwise multiple comparison procedure (Dunn's test) (Zar 1996).

## 3.3 Results

### 3.3.1 DNA damage

#### 3.3.1.1 Gills

Gills of fish exposed to both concentrations of Roundup® (58 and 116 $\mu g.L^{-1}$) demonstrated an increase in GDI values, after 1- and 3-day exposures, when compared to respective control (Fig. 1). Concerning 1-day exposure, gills GDI presented a 1.6 and 1.7 times increase, respectively for 58 and 116 $\mu g.L^{-1}$ Roundup® concentrations, when compared to control. After 3 days, GDI values presented a 1.4 and 1.8 times increase, respectively for 58 and 116 $\mu g.L^{-1}$ groups. Moreover, the 116 $\mu g.L^{-1}$ group displayed a significant GDI increase, when compared with the 58 $\mu g.L^{-1}$ group. Globally, the GDI showed to be concentration dependent, whereas only one time-related alteration was noticeable, concerning a decrease from 1- to 3-day exposure for the concentration 58 $\mu g.L^{-1}$ 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 as well as than both concentrations of Roundup®.

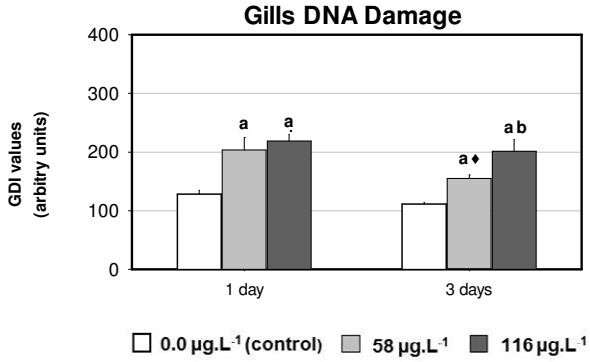


**Fig. 1.** Mean values of genetic damage indicator (GDI), expressed as arbitrary units, measured by comet assay in gills of *A. anguilla* exposed to 58 and 116 µg.L$^{-1}$ Roundup®, during 1 and 3 days. Statistically significant differences ($p<0.05$) are: (a) vs. control and (b) vs. 58 µg.L$^{-1}$ (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. Hence, 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 116 µg.L$^{-1}$ group significant increases were detected in classes 2, 3 and 4. Sub-total of damaged nucleoids (sum of damage classes 2, 3 and 4) showed increments of 3 and 3.5 times, respectively for 58 and 116 µg.L$^{-1}$ groups, when compared to control, highlighting an influence of Roundup® concentration in the magnitude of damage. Following 3-day exposure to 58 µg.L$^{-1}$ of Roundup®, only class 2 showed a significant increase in comparison to 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). Damaged nucleoids (sub-total 2+3+4) frequency was significantly elevated in both treated groups (4.7 and 6.6 times, respectively for 58 and 116 µg.L$^{-1}$), though it seems to decrease in comparison with the corresponding levels after 1-day exposure (significantly lower for 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.

**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).

| | | Gills DNA Damage Classes | | | | | |
|---|---|---|---|---|---|---|---|
| Exposure Time | Roundup® Concentration (µg.L$^{-1}$) | 0 | 1 | 2 | 3 | 4 | Sub-Total (2+3+4) |
| 1 day | 0.0 (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 | 0.0 (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] |

3.3.1.2 Liver

After 1-day exposure, liver of fish treated with both Roundup® concentrations (Fig. 2A) displayed significantly higher GDI values, in relation to control. The increments were around 1.5 and 1.6 times, respectively for 58 and 116 µg.L$^{-1}$. In what concerns to 3-day exposure, only the higher concentration showed a significant GDI increase (1.6 times), when compared to control. This group also displayed a significant increase in relation to 58 µg.L$^{-1}$ group (1.7 times). Considering the GDI results as a whole, a concentration dependency was not clear. Differently, time-related variations included a significant decrease in 58 µg.L$^{-1}$ group and an increase in 116 µg.L$^{-1}$ group. The positive control displayed an average GDI of 283.0 (± 11.80) arbitrary units, showing to be significantly higher than the negative control as well as than both concentrations of Roundup®.

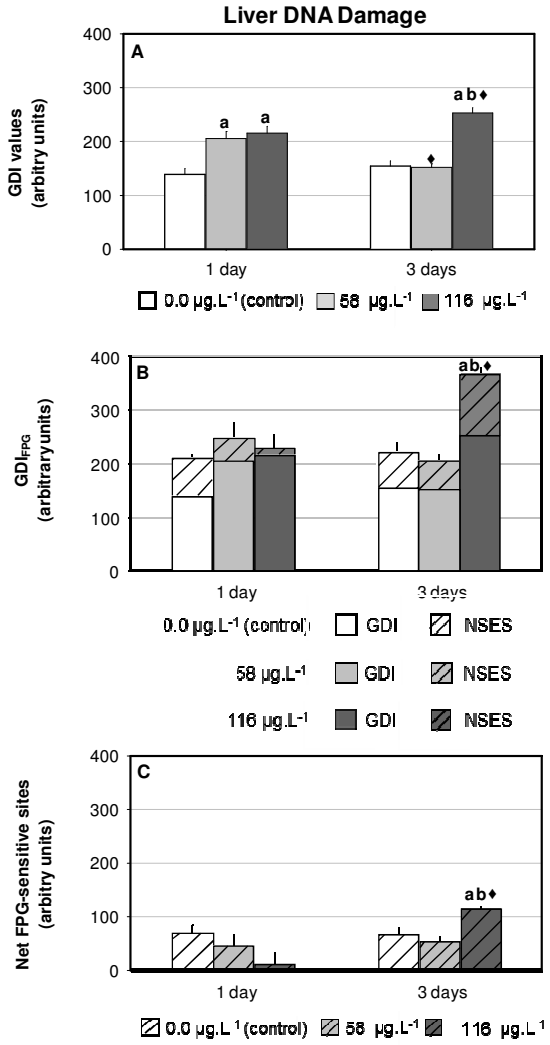


**Fig. 2.** Mean values of DNA damage, expressed in arbitrary units, measured by comet assay in liver of *A. anguilla* exposed to 58 and 116 µg.L$^{-1}$ Roundup®, during 1 and 3 days. (A) Genetic damage indicator (GDI) after standard (alkaline) comet assay. Values after enzyme-modified comet assay, as 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.

Concerning the frequency of individual classes of damage (Table 2), after 1-day exposure to 58 µg.L$^{-1}$, none of the classes displayed significant increases; however, a significant increase (about 1.7 times) was found in sub-total of damaged nucleoids for this group in relation to control. On the other hand, 116 µg.L$^{-1}$ group exhibited significant increases (compared with the control) either in classes 3 and 4 or in sub-total of damaged

nucleoids (about 1.6 times). Both Roundup® concentrations showed a significant decrease of class 1 frequency, in relation to the control. The results of 3-day exposure revealed significant alteration in relation to the control only for the higher herbicide concentration. Thus, 116 $\mu g.L^{-1}$ group showed significantly higher frequency of classes 3 and 4 as well as sub-total of damaged nucleoids in relation to control and to 58 $\mu g.L^{-1}$ groups.

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

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

**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 $\mu g.L^{-1}$ Roundup®, during 1 and 3 days. Statistically significant differences ($p<0.05$) are: (a) vs. control and (b) vs. 58 $\mu g.L^{-1}$ (within the same exposure duration); (♦) vs. 1-day exposure (for the same exposure condition).

| | | Liver DNA Damage Classes | | | | | |
|---|---|---|---|---|---|---|---|
| Exposure Time | Roundup® Concentration ($\mu g.L^{-1}$) | 0 | 1 | 2 | 3 | 4 | Sub-Total (2+3+4) |
| 1 day | 0.0 (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±0.29 | 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 | 0.0 (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$^{ab♦}$ |

### 3.3.2 Antioxidant responses

#### 3.3.2.1 Gills

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

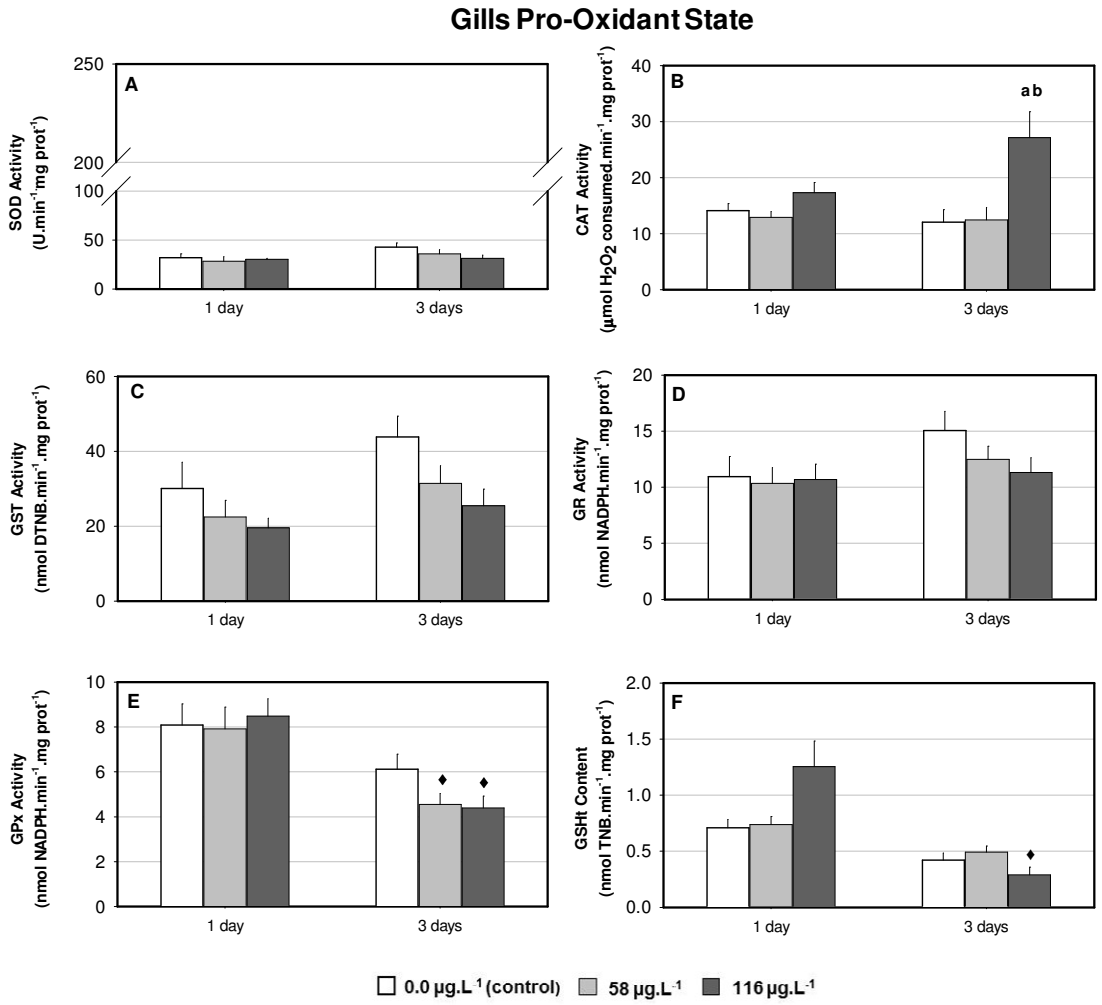


**Fig. 3.** Mean superoxide dismutase (SOD) (A), catalase (CAT) (B), glutathione-S-transferase (GST) (C), glutathione reductase (GR) (D) and glutathione peroxidase (GPx) (E) activities, as well as total glutathione (GSHt) content (F) in gills of *A. anguilla* exposed to 58 and 116 µg.L$^{-1}$ Roundup®, 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.

3.3.2.2 Liver

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

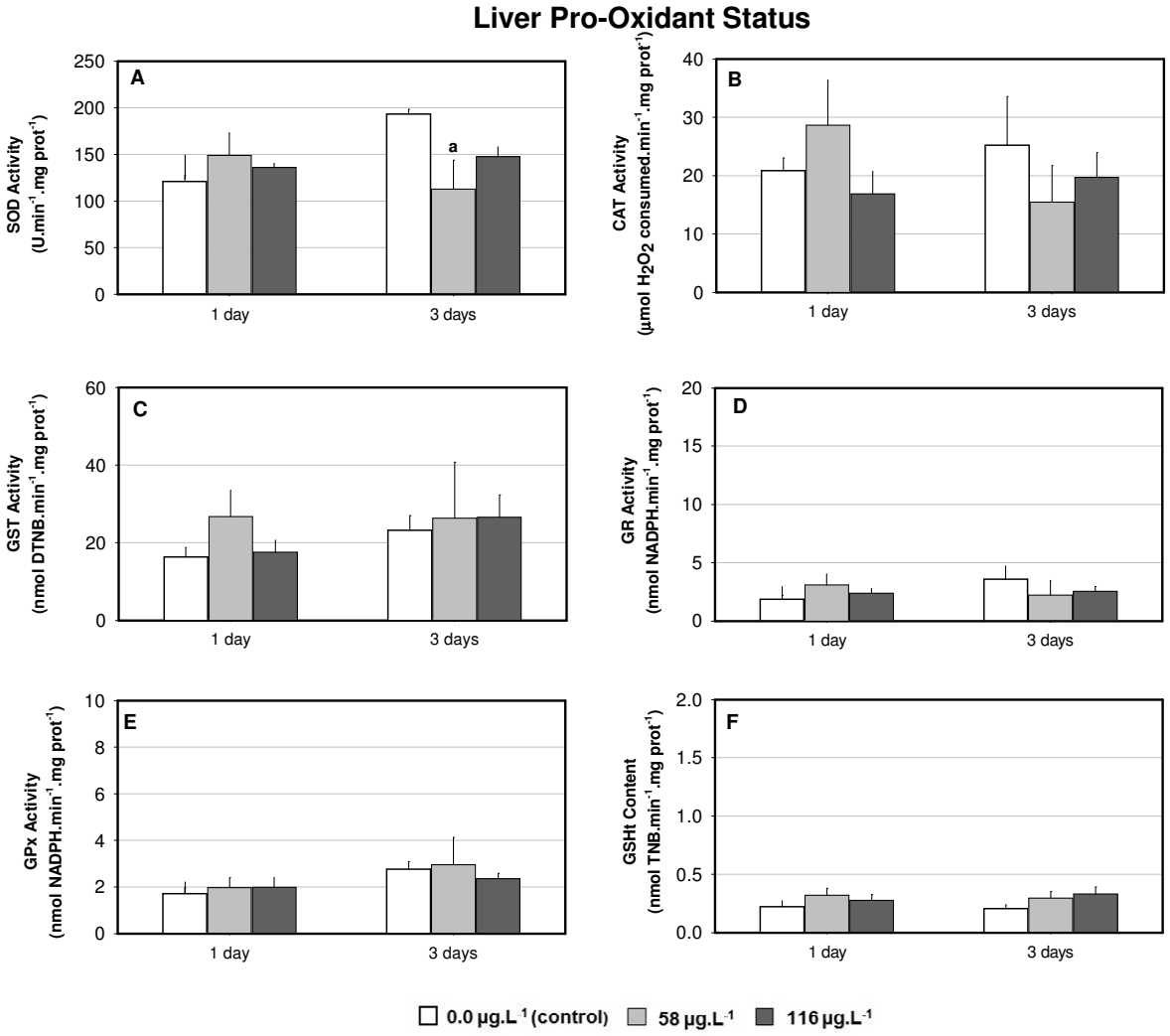


**Fig. 4.** Mean superoxide dismutase (SOD) (A), catalase (CAT) (B), glutathione-S-transferase (GST) (C), glutathione reductase (GR) (D) and glutathione peroxidase (GPx) (E) activities, as well as total glutathione (GSHt) content (F) in liver of *A. anguilla* exposed to 58 and 116 µg.L$^{-1}$ Roundup®,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

## 3.4 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 (Giesy et al.

2000). Giesy et al. (2000) developed a model to estimate the worst-case exposure conditions. Taking into account this theoretical model, values within 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 estimations have proven to be correct, since concentrations of glyphosate were detected in the range 75-90 µg.$L^{-1}$ in the Orge watershed (France) (Botta et al. 2009) and higher levels (0.5-1.0 mg.$L^{-1}$) than those predicted were sporadically found following direct application to water (WHO 1994). Extreme values of glyphosate were found near agricultural areas, in Brazil, corresponding to the range 0.36-2.16 mg.$L^{-1}$ of commercial formulation Roundup® (360 g.$L^{-1}$ glyphosate) (Rodrigues and Almeida 2005).

Accordingly, the concentrations tested in the current research (corresponding to 18 and 36 µg.$L^{-1}$ of glyphosate) are realistic, contrasting with other studies where Roundup® concentrations were one order of magnitude higher (Jiraungkoorskul et al. 2003; Çavas and Könen 2007; Cavalcante et al. 2008; Langiano and Martinez 2008; Lushchak et al. 2009). In addition, the present study represents an important progress in relation to the (few) previous fish studies on Roundup® genotoxicity by its mechanistic approach and by the exploration of organ-specific susceptibilities. To the authors' knowledge, a single report is available on gills in this context (Cavalcante et al. 2008) and no studies were yet performed in liver. Furthermore, the use of 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 (Akcha et al. 2003), has never been used before to assess pesticide-induced DNA damage.

### 3.4.1 Gills DNA damage and pro-oxidant state

GDI results clearly indicated the Roundup® potential to induce DNA strand breaks in branchial cells, in both tested concentrations (58 and 116 µg.$L^{-1}$) and exposure times (1 and 3 days). Overall, a concentration-dependence was observed mainly after 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 to day 1, though still higher than the respective control), revealing a concentration-related pattern. This temporal variation can be explained by the lowering levels of the pesticide (or their metabolites) in gills tissue, combined with the intervention of DNA-repair system and/or cell turnover. In this direction, it was demonstrated that gills epithelium is regularly subject to exfoliation and erosion, which is counteracted by an intense cell division rate (Pacheco et al. 1993).

Examining the individual damage classes, it was possible to identify in treated fish, invariably, decreases in class 1 (not damaged nucleoids) frequency, whenever damaged nucleoids classes (2, 3 and 4) predominantly presented increased frequencies. Despite in line with GDI data, it is important to highlight the damaged nucleoids (sub-total 2+3+4) frequency, since it exhibited higher increments in treated groups (maximum 6.6 times in relation to control) when compared to the corresponding increments obtained for GDI values (maximum 1.7 times). Rather than GDI, 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. (2008) in gills of the neotropical fish *Prochilodus lineatus*, where the Roundup® potential 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 have been addressed (Glusczak et al. 2007; Langiano and Martinez 2008; Lushchak et al. 2009; Menezes et al. 2010). 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. Though Roundup® has been shown as an oxidative stress agent on different fish organs/tissues (Langiano and Martinez 2008; Lushchak et al. 2009; Menezes et al. 2010), its impact specifically on gills pro-oxidant state 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 (Ahmad et al. 2004; Santos et al. 2006). Therefore, DNA oxidation was hypothesised as a potential kind of damage induced by Roundup® in branchial cells. However, present data concerning 1-day exposure revealed that DNA strand breaks induction was not accompanied by an increased pro-oxidant state, suggesting that DNA was not oxidatively damaged under these conditions. Differently, after 3-day exposure, the higher concentration (116 $\mu g.L^{-1}$) 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 the most toxic ROS. Hence, under these circumstances, the DNA oxidation may play a role on the Roundup® genotoxic capacity demonstrated in *A. anguilla* gills.

### 3.4.2 Liver DNA damage and underlying mechanisms

Following 1-day exposure, GDI results demonstrated that Roundup® affects DNA integrity of hepatic cells in 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 on the liver DNA damaging effect of Roundup® was attempted combining the analysis of antioxidant responses and the identification of additional DNA breaks corresponding to FPG-sensitive sites. Hence, antioxidant system did not sign 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. (2009) who observed that an intraperitoneal administration of glyphosate in mice caused genotoxicity in liver, despite the absence of antioxidant defences induction. 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 116 $\mu g.L^{-1}$ group after 1-day exposure, highlighting that under short exposures the base oxidation is not a relevant mechanism of damage. Differently, following 3-day exposure, oxidised purines were found to be elevated in the 116 $\mu g.L^{-1}$ group, as depicted in the significant increase of $GDI_{FPG}$ and net FPG-sensitive sites observed in comparison to control and 58 $\mu g.L^{-1}$ groups. Surprisingly, it should be noted that the Roundup-induced DNA oxidative damage (signalized by the enzyme-modified comet assay) was not accompanied by an activation of the antioxidant system. Thus, as previously stated (Ahmad et al. 2006), 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 mass antioxidants is counterbalanced by *de novo* synthesis and/or inhibitory actions impair the activity of enzymatic antioxidants. Taking into account the present results, the occurrence of this effect cannot be excluded, namely in the light of the SOD activity decrease detected after 3-day exposure to 58 $\mu g.L^{-1}$. Giving support to this observation, Lushchak et al. (2009) found that Roundup® exposure (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 notice 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 toxicity expression as enzyme

inhibition or as DNA damage. In agreement with Modesto and Martinez (2010a, 2010b), who found an activity decrease in antioxidant enzymes (SOD, CAT, GST, and GPX) of fish exposed to Roundup®, the present results point to the enzymatic inhibition as a potential mechanism through which this herbicide can induce oxidative stress.

Saleha Banu et al. (2001) 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 (Wild 1975) affecting DNA bases either directly or indirectly via protein alkylation (Mohan 1973; Green et al. 1974). A study performed in mice, also showed the ability of Roundup® to induce a dose-dependent formation of DNA adducts (Peluso et al. 1998). Therefore, the previously invoked mechanisms (ROS-independent processes) played a key role on the generation of DNA damage in hepatic cells under short exposures (1 day), while for 3-day exposure (116 $\mu g.L^{-1}$) DNA bases oxidation appears as a relevant mechanism of damage.

### 3.4.3 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 3-day exposure to 58 $\mu g.L^{-1}$. Nevertheless, it was also notorious an organ-specificity as GDI values in liver returned to the control level whereas in gills remained significantly higher than 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 studied organs concerned the time-related increase in GDI levels, only observed in liver (116 $\mu g.L^{-1}$).

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

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

### 3.5 Conclusions

The present findings clearly demonstrated the genotoxic properties of Roundup® expressed as DNA strand breaks (measured by comet assay) in gills and liver cells of *A. anguilla* exposed to realistic concentrations. Therefore, it was pointed out the risk to ichthyopopulations 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 tested concentration and exposure duration. Hence, after 1-day exposure, it was demonstrated that an increase on pro-oxidant state is not a compulsory condition for the induction of DNA damaging effects of Roundup®. Nevertheless, by increasing the exposure length (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 FPG-sensitive sites (signalling oxidatively altered DNA bases) elevation detected in liver through 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 in the presence of the lower concentration (58 $\mu g.L^{-1}$).

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**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.

The authors declare that there are no conflicts of interest.

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# Chapter IV

**Differential genotoxicity of Roundup® formulation and its constituents in blood cells of fish (*Anguilla anguilla*): considerations on chemical interactions and DNA damaging mechanisms**

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# 4 Differential genotoxicity of Roundup® formulation and its constituents in blood cells of fish (*Anguilla anguilla*) – considerations on chemical interactions and DNA damaging mechanisms

**Abstract**

It has been widely recognized that pesticides represent a potential threat in aquatic ecosystems. However, the knowledge on the genotoxicity of pesticides to fish is still limited. Moreover, genotoxic studies have been almost exclusively focused on the active ingredients, whereas the effect of adjuvants is frequently ignored. Hence, the present study addressed the herbicide Roundup®, evaluating the relative contribution of the active ingredient (glyphosate) and the surfactant (polyethoxylated amine; POEA) to the genotoxicity of the commercial formulation on *Anguilla anguilla*. Fish were exposed to equivalent concentrations of Roundup® (58 and 116 $\mu g.L^{-1}$), glyphosate (17.9 and 35.7 $\mu g.L^{-1}$) and POEA (9.3 and 18.6 $\mu g.L^{-1}$), during 1 and 3 days. The comet assay was applied to blood cells, either as the standard procedure, or with an extra step involving DNA lesion-specific repair enzymes in an attempt to clarify DNA damaging mechanisms. The results confirmed the genotoxicity of Roundup®, also demonstrating the genotoxic potential of glyphosate and POEA individually. Though both components contributed to the overall genotoxicity of the pesticide formulation, the sum of their individual effects was never observed, pointing out an antagonistic interaction. Although POEA is far from being considered biologically inert, it did not increase the risk associated to glyphosate when the two were combined. The analysis of oxidatively induced breaks suggested that oxidation of DNA bases was not a dominant mechanism of damage. The present findings highlighted the risk posed to fish populations by the assessed chemicals, jointly or individually, emphasizing the need to define regulatory thresholds for all the formulation components and recommending, in particular, the revision of the hazard classification of POEA.

**Keywords**: Roundup®; glyphosate; POEA; genotoxicity; DNA lesion-specific repair enzymes; fish.

## 4.1 Introduction

Herbicides represent a hazardous and widely spread group of environmental contaminants, affecting non-target organisms, namely fish, since they can easily reach the aquatic systems by runoff, drainage, leaching or inadvertent aerial overspray. The formulations of these agrochemicals typically include the active ingredient and one or more adjuvants (Foy 1987) that are meant to aid or modify the active ingredient function (Valkenburg 1982). In this framework, surfactants are the most commonly incorporated. Paradoxically, toxicity evaluation is almost exclusively focused on the active ingredient, whereas the effects of the different adjuvants, either directly or indirectly, are a topic largely ignored by environmental toxicologists and protection agencies. Moreover, adjuvants are generally considered "dilutants" or "inerts" for regulatory purposes (Richard et al. 2005) and risk assessment usually fails to look at the effects resulting from interactions with the active ingredient (Renner 2005).

The broad-spectrum herbicide Roundup® appears as one of the most popular commercial formulations worldwide, with a wide use in agriculture, ornamental gardens and for aquatic weed control (Giesy et al. 2000). Roundup® is a combination of glyphosate, as active ingredient, and a non-ionic surfactant that promotes the penetration of glyphosate into the plant cuticle - polyethoxylated amine (POEA) (Relyea 2005; Brausch and Smith 2007). Glyphosate has been considered as practically nontoxic to birds and mammals and moderately toxic to practically nontoxic to fish and invertebrates (USEPA 1993). However, glyphosate-based formulations are generally considered more toxic than pure glyphosate (Rank et al. 1993; Tsui and Chu 2003; Peixoto 2005). Formulations containing POEA are of particular interest in ecotoxicology since they are known to exhibit relatively higher toxicity to many aquatic organisms, as compared to glyphosate alone or other glyphosate-based formulations without this particular surfactant (Perkins et al. 2000; Howe et al. 2004). It is believed that POEA disrupts cell membranes on respiratory surfaces (Lindgren et al. 1996a; Relyea 2005) and its toxicity has been demonstrated for crustaceans (Brausch and Smith 2007; Brausch et al. 2007; Frontera et al. 2011) and amphibians (Relyea 2005). Nevertheless, the toxicity of POEA to fish has not yet been described.

Since genotoxicity is one of the most adverse effects of contaminants, including pesticides, on wildlife, it is noteworthy that little is known about the risk of DNA damage associated with fish exposure to Roundup® or its components. Although the available literature has recently demonstrated the genotoxicity of the commercial formulation in fish (Grisolia 2002; Çavas and Könen 2007; Cavalcante et al. 2008; Guilherme et al. 2010),

the action of the active ingredient alone and the comparison of its genotoxic potential within the commercial mixture remain unknown. Furthermore, nothing is known about the contribution of POEA to the overall genotoxic potential of Roundup®.

Keeping in mind the knowledge gaps identified, the main goal of the present research was to evaluate the relative contribution of the active ingredient (glyphosate) and the surfactant (POEA) to the genotoxicity of the commercial formulation Roundup® on fish. The classification of POEA as "inert of minimal concern" will also be called into question. Therefore, the comet assay was used to detect DNA damage in blood cells of juvenile *Anguilla anguilla*, following a short-term exposure to the previous agents, adopting environmentally realistic concentrations. With the purpose to shed light on the 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. This methodology allows the detection of a genotoxic risk resulting from unspecific (alkali-labile sites, DNA-DNA/DNA-protein cross-linking, and single strand breaks associated with incomplete excision repair sites) and specific (bases oxidation) damage. Hence, it may clarify if the induced damage has an oxidative cause. The European eel (*A. anguilla*) was adopted as test species due to its sensitivity and suitability as bioindicator. As demonstrated in previous studies (Guilherme et al. 2010, 2012), this species showed to be able of reflecting the pernicious effects of pesticides, namely Roundup®.

## 4.2 Material and Methods

### 4.2.1 Chemicals

The experiment was conducted using the commercial formulation 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 POEA (16 %) as surfactant. Glyphosate was obtained from Sigma-Aldrich Chemical Company (Spain). POEA (solution at 785 $g.L^{-1}$) was kindly provided by Professor Robert Bellé (UMR 7150 CNRS/ UPMC, Station Biologique de Roscoff, France). DNA lesion-specific repair enzymes, namely formamidopyrimidine DNA glycosylase (FPG) and endonuclease III (EndoIII) were purchased from Professor Andrew Collins (University of Oslo, Norway). All the other chemicals needed to perform comet assay were obtained from the Sigma-Aldrich Chemical Company (Spain).

4.2.2 Test animals and experimental design

European eel (*Anguilla anguilla* L.) specimens with an average weight 0.25±0.02 g (glass eel stage) were captured from Mondego river mouth, Figueira da Foz, Portugal. Eels were acclimated to the 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 physico-chemical conditions: salinity 0, temperature 20±1ºC, pH 7.3±0.2, nitrate 25±0.5 $mg.L^{-1}$, nitrite 0.05±0.01 $mg.L^{-1}$, ammonia 0.1±0.01 $mg.L^{-1}$, dissolved oxygen 8.1±0.5 $mg.L^{-1}$. During this period, fish were daily fed with fish roe.

The experiment was carried out in 1-L aquaria, in a static mode, under the conditions described for the acclimation period. After acclimation, 168 eels were divided into fourteen groups, corresponding to seven test conditions and two exposures times (7x2). Thus, fish were exposed to 58 and 116 $\mu g.L^{-1}$ Roundup® (groups R1 and R2, respectively), 17.9 and 35.7 $\mu g.L^{-1}$ glyphosate (groups G1 and G2, respectively), and 9.3 and 18.6 $\mu g.L^{-1}$ POEA (groups P1 and P2, respectively). Another group was kept with clean water - 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 concentrations of Roundup® assayed are considered environmentally relevant (Guilherme et al. 2010). Taking this as a starting point, the exposure concentrations of glyphosate and POEA were calculated on a proportionality basis, considering the formulation described in point 2.1. (for instance, it was assumed that 58 µg of the formulation contains 17.9 µg of glyphosate and approximately of 9.3 µg of POEA). Stock solutions of each compound were prepared (in deionised 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 4 fish for each condition/time (3x4 = 12 fish).

Fish were not fed during the experimental period. Fish were sacrificed by cervical transaction at the post-opercular region and blood collected from the heart using heparinised capillary tubes. Two µL of blood were immediately diluted in 1 mL of ice-cold phosphate-buffered saline (PBS) to prepare a cell suspension, which was kept on ice until further procedure.

4.2.3 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 the assay procedure with the 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), in order to increase the assay output. Briefly, 20 µL of cell suspension (previously prepared in PBS) were mixed with 70 µL of 1% low melting point agarose (in PBS). Eight drops of 6 µL were placed onto the precoated slide as two rows of 4 (4 groups of 2 replicates), without coverslips, each drop/gel containing approximately 1500 cells. The gels were left for ±5 min at 4 ºC in order to solidify the 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 ºC, for 1 h. After lysis of agarose-embedded cells, slides were washed 3 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 ºC.

Three sets of slides were prepared: two sets were incubated with endonucleases (1) FPG and (2) EndoIII, that convert oxidised 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 were applied in each gel, along with a coverslip, prior to incubation at 37 ºC 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 $\mu g.mL^{-1}$).

Slides with eight gels each, and 50 nucleoids per gel, were observed, using a Leica DMLS fluorescence microscope (400x 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 this formula:

GDI = [(% nucleoids class 0)×0] + [(% nucleoids class 1)×1] + [(% nucleoids class 2)×2] + [(% nucleoids class 3)×3] + [(% nucleoids class 4)×4]

GDI values were expressed as arbitrary units in a scale of 0 to 400 per 50 scored nucleoids (as average value for the 2 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 designated $GDI_{FPG}$ and $GDI_{EndoIII}$, respectively. Additional DNA breaks corresponding to net enzyme-sensitive sites alone ($NSS_{FPG}$ and $NSS_{EndoIII}$) were also expressed. In order to better estimate the overall magnitude of oxidative DNA damage, the sum of GDI with additional DNA breaks corresponding to both net FPG-($NSS_{FPG}$) and EndoIII-sensitive sites ($NSS_{EndoIII}$) was also calculated ($GDI_{FPG+EndoIII}$).

Besides GDI scores, the frequency of nucleoids observed in each comet class was also expressed, as recommended by Azqueta et al. (2009). In order to improve the expression of the DNA damage extent (Palus et al. 1999; Çavas and Könen 2007), the sub-total frequency of nucleoids with medium (class 2), high (class 3) and complete (class 4) damaged DNA was also calculated (2+3+4).

#### 4.2.4 Statistical analysis

Statistica 7.0 software was used for statistical analysis. All data were first tested for normality and homogeneity of variance to meet statistical demands. One-way Analyses of Variance (ANOVA), followed by a post-hoc Dunnett's test, was applied to compare the different treatment 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 between them. The Tukey test was applied as a post-hoc comparison. In all the analyses, differences between means were considered significant when $p<0.05$ (Zar 1996).

## 4.3 Results

### 4.3.1 Non-specific DNA damage

Analyzing GDI values after 1 day exposure (Fig. 1), it was observed that all the treatments, with the exception of the lower concentration of Roundup® (1R1), displayed significantly higher values in comparison with the control. Comparing the exposure to Roundup® with the exposure to its ingredients, the results demonstrated that only the lower glyphosate concentration (1G1) presented a significant GDI increase when compared with the equivalent concentration of Roundup®.

In relation to 3 days exposure, significant increases in GDI values (Fig. 1) in relation to control were observed for 3R2, 3G1 and both POEA exposed groups (3P1 and 3P2). Comparing the effect of each the Roundup® ingredients, POEA increased GDI values when compared to glyphosate in the higher concentrations (3P2 *vs*. 3G2).

No concentration related differences were observed in any exposure duration. On the other hand, the analysis of temporal variations of GDI values demonstrated a time-related decrease for the higher concentration of glyphosate (1G2 *vs*. 3G2).

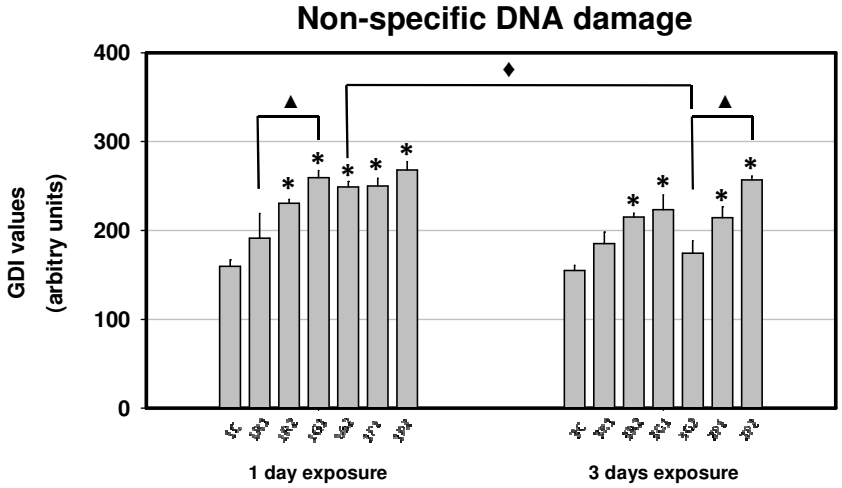


**Fig 1.** Mean values of genetic damage indicator (GDI) measured by the standard (alkaline) comet assay in blood cells of *A. anguilla* exposed to 58 and 116 µg.L$^{-1}$ Roundup® (R1, R2), 17.9 and 35.7 µg.L$^{-1}$ glyphosate (G1, G2) or 9.3 and 18.6 µg.L$^{-1}$ polyethoxylated tallowamine (POEA; P1, P2), 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; (▲) between treatments, within the same exposure time; (♦) between exposure times, within the same treatment.

Considering the results in terms of individual DNA damage classes (Table 1), and in what concerns to 1 day exposure, it was perceptible that in 1R1 group, like in the control, the predominant classes were 1 and 2. Differently, all the other treatment groups presented higher frequencies in classes 2 and 3. In addition, 1P2 showed to be the group where class 4 showed the highest prevalence (though not significantly different from the control). The sub-total of damaged nucleoids (2+3+4) revealed significantly higher values in all treatment groups in relation to the control, except 1R1. After 3 days exposure (Table 1), control, 3R1 and 3G2 groups displayed 1 and 2 as the most frequent damage classes, while all the other groups showed classes 2 and 3 as the most predominant. The sub-total of damaged nucleoids revealed significantly higher values, in relation to the control, for 3R2, 3G1, 3P1 and 3P2 groups. Comparing 1 and 3 days results displayed in table 1, a general time-related decrease was observed in the frequency of class 3 (with significant

differences for G2 and P1), whereas an opposite temporal variation pattern was observed for classes 1 and 2 (with a significant difference for class 1 in G2).

**Table 1.** Mean frequencies (%) of each DNA damage class and sub-total of damaged nucleoids (± standard error), measured by the standard (alkaline) comet assay, in blood cells of *A. anguilla* exposed to 58 and 116 µg.L$^{-1}$ Roundup® (R1, R2), 17.9 and 35.7 µg.L$^{-1}$ glyphosate (G1, G2) or 9.3 and 18.6 µg.L$^{-1}$ polyethoxylated tallowamine (POEA; P1, P2), during 1 and 3 days (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), within the same exposure time; letters between treatments, within the same exposure time - (a) vs. R1, (b) vs. R2, (d) vs. G2 and (e) vs. P1; (♦) between exposure times, within the same treatment.

| | | DNA Damage Classes | | | | | |
|---|---|---|---|---|---|---|---|
| | Exposure Conditions | 0 | 1 | 2 | 3 | 4 | Sub-total (2+3+4) |
| 1 day | 1C | 5.20±5.20 | 38.11±4.31 | 49.03±3.18 | 7.44±1.59 | 0.22±0.22 | 56.69±3.21 |
| | 1R1 | 0.00±0.00 | 27.33±2.26 | 50.78±3.10 | 19.67±4.17 | 2.22±1.36 | 72.67±8.52 |
| | 1R2 | 0.00±0.00 | 9.67±2.26 * | 53.00±3.10 | 34.92±4.17 * | 2.42±1.36 | 90.33±2.26 * |
| | 1G1 | 0.00±0.00 | 1.08±0.63 *a | 43.58±2.68 | 50.33±2.83 *a | 5.00±1.63 | 98.92±0.63 * |
| | 1G2 | 0.00±0.00 | 0.92±0.62 * | 50.58±3.10 | 47.00±3.29 * | 1.50±0.82 | 99.08±0.62 * |
| | 1P1 | 0.00±0.00 | 4.23±1.99 * | 44.92±2.35 | 48.51±3.11 *a | 2.33±0.65 | 95.77±1.99 * |
| | 1P2 | 0.00±0.00 | 0.25±0.25 * | 39.08±2.87 | 52.75±2.37 * | 7.92±2.35 | 99.75±0.25 * |
| 3 days | 3C | 0.00±0.00 | 49.75±3.10 | 45.58±2.63 | 4.67±1.17 | 0.00±0.00 | 50.25±3.10 |
| | 3R1 | 0.25±0.25 | 32.71±4.62 | 49.88±2.76 | 15.92±2.13 | 1.25±0.74 | 67.04±4.76 |
| | 3R2 | 0.00±0.00 | 12.58±2.53 * | 62.38±3.20 | 23.53±3.79 * | 1.50±0.56 | 87.42±2.53 * |
| | 3G1 | 0.00±0.00 | 8.73±2.88 * | 56.82±2.45 | 31.64±3.73 * | 2.82±1.24 | 91.27±8.05 * |
| | 3G2 | 0.00±0.00 | 36.44±5.71 ♦ | 52.68±3.84 | 11.24±2.54 ♦ | 0.00±0.00 | 63.92±5.83 ♦ |
| | 3P1 | 0.00±0.00 | 13.83±2.35 * | 59.75±2.05 | 24.67±3.58 *♦ | 1.75±0.90 | 86.17±2.35 * |
| | 3P2 | 0.00±0.00 | 1.50±0.51 *d | 43.92±2.66 | 51.17±2.64 *bde | 3.42±1.36 | 98.50±0.51 *d |

Table 2 (3-way ANOVA results) revealed a significant effect of the factors agent and time on GDI levels, as well as a significant interaction agent x concentration.

**Table 2.** Results of three-way ANOVA testing the effect of agent, concentration and time, as well as the interactions between them (agent x concentration, agent x time, concentration x time and agent x time x concentration) on the levels of DNA damage in blood cells of *A. anguilla* exposed to Roundup®, glyphosate or polyethoxylated tallowamine (POEA), during 1 and 3 days. Both F and p values are given for each variable. Non significant differences are signalized as "ns".

| Parameter | Factors | | | | | | Interactions | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Agent | | Concentration | | Time | | Agent x Concentration | | Agent x Time | | Concentration x Time | | Agent x Time x Concentration | |
| | *F* | *p* | *F* | *p* | *F* | *p* | *F* | *p* | *F* | *p* | *F* | *p* | *F* | *p* |
| **GDI** | 11.10 | 0.0004 | 2.56 | ns | 16.97 | 0.0004 | 8.21 | 0.0019 | 3.35 | ns | 0.28 | ns | 1.59 | ns |
| **$GDI_{FPG}$** | 3.61 | 0.0425 | 2.39 | ns | 6.23 | 0.0198 | 4.09 | 0.0296 | 0.31 | ns | 0.04 | ns | 0.16 | ns |
| **$NSS_{FPG}$** | 12.21 | 0.0002 | 0.00 | ns | 4.81 | 0.0383 | 5.53 | 0.0106 | 3.58 | 0.0437 | 1.00 | ns | 2.65 | ns |
| **$GDI_{EndoIII}$** | 11.52 | 0.0003 | 5.64 | 0.0259 | 11.91 | 0.0021 | 6.77 | 0.0047 | 6.36 | 0.0061 | 0.89 | ns | 2.17 | ns |
| **$NSS_{EndoIII}$** | 1.74 | ns | 0.03 | ns | 5.24 | 0.0312 | 2.32 | ns | 0.42 | ns | 2.21 | ns | 3.56 | 0.0444 |
| **$GDI_{FPG+EndoIII}$** | 4.50 | 0.0219 | 2.72 | ns | 0.48 | ns | 1.75 | ns | 0.73 | ns | 2.21 | ns | 3.88 | 0.0346 |

### 4.3.2 Oxidative DNA damage

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

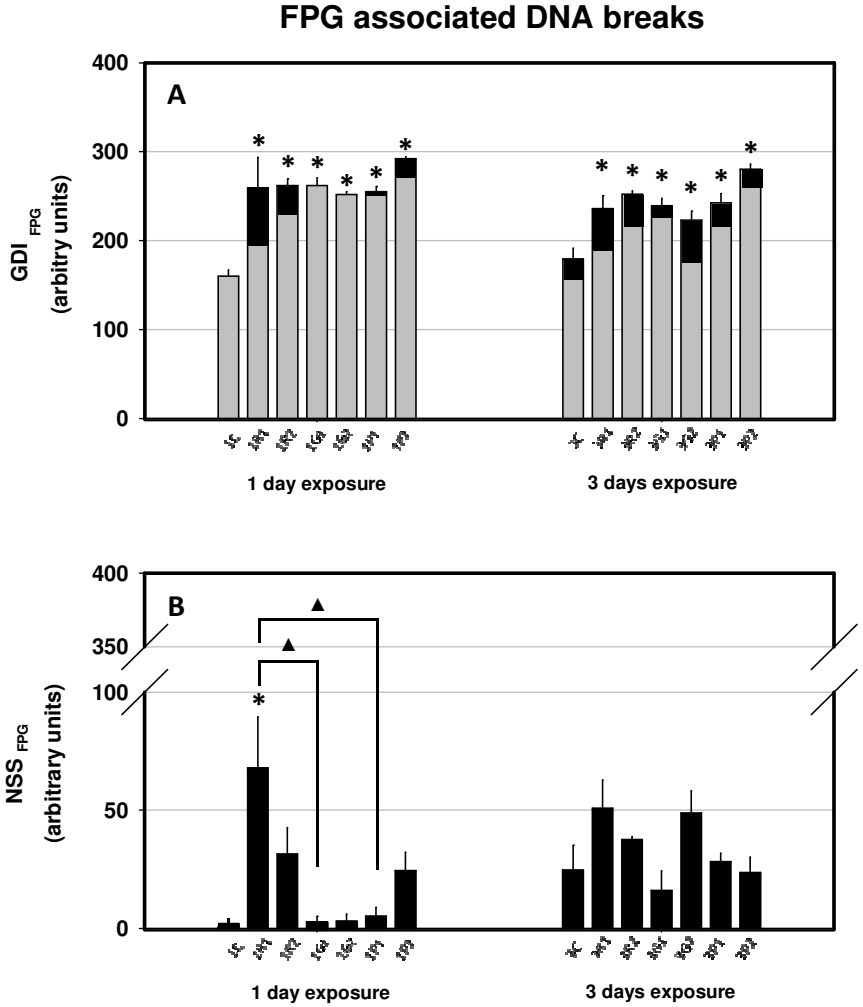


**Fig. 2.** Mean values of DNA damage, measured by comet assay in blood cells of *A. anguilla* exposed to 58 and 116 µg.L$^{-1}$ Roundup® (R1, R2), 17.9 and 35.7 µg.L$^{-1}$ glyphosate (G1, G2) or 9.3 and 18.6 µg.L$^{-1}$ polyethoxylated tallowamine (POEA; P1, P2), during 1 and 3 days (in the abbreviations 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 oxidised purine bases: (A) overall damage ($GDI_{FPG}$) and partial scores, namely genetic damage indicator (GDI; grey) 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.

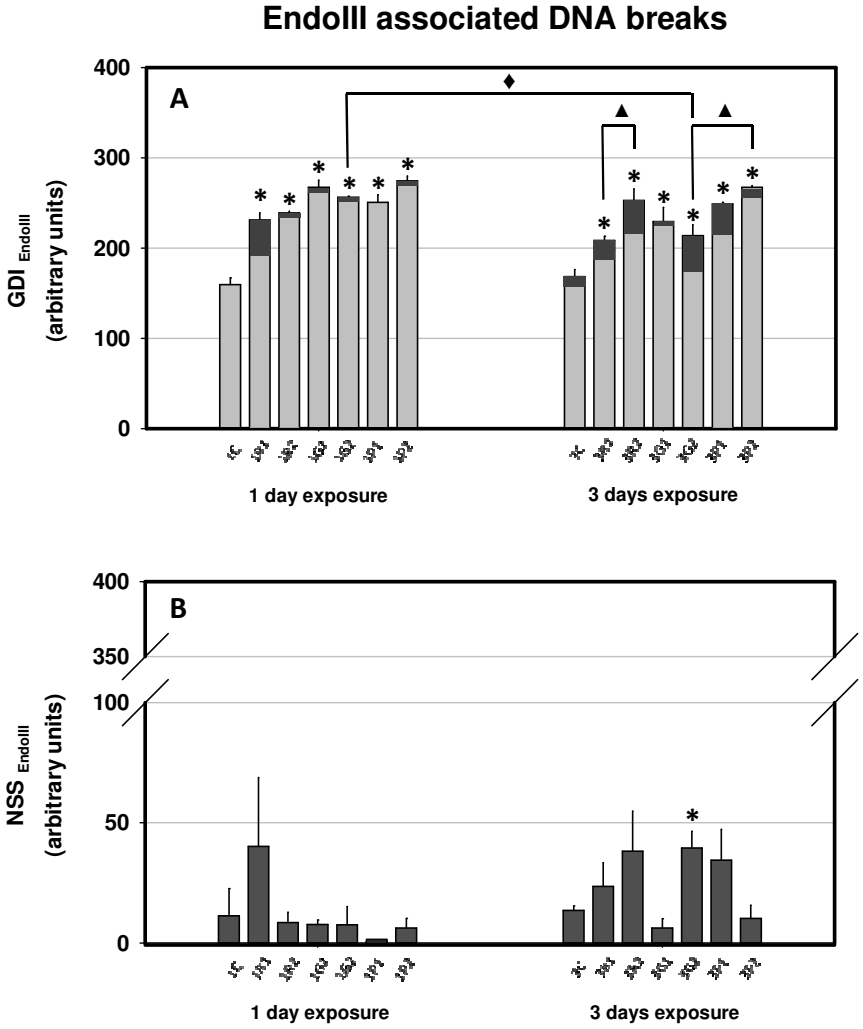


**Fig. 3.** Mean values of DNA damage, measured by comet assay in blood cells of *A. anguilla* exposed to 58 and 116 µg.L$^{-1}$ Roundup® (R1, R2), 17.9 and 35.7 µg.L$^{-1}$ glyphosate (G1, G2) or 9.3 and 18.6 µg.L$^{-1}$ polyethoxylated tallowamine (POEA; P1, P2), during 1 and 3 days (in the abbreviations 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 oxidised pyrimidine bases: (A) overall damage ($GDI_{EndoIII}$) and partial scores, namely genetic damage indicator (GDI; light grey) and additional DNA breaks corresponding to net EndoIII-sensitive sites ($NSS_{EndoIII}$; dark grey); (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; (▲) between treatments, within the same exposure time; (♦) between exposure times, within the same treatment.

*FPG associated DNA breaks*

Concerning 1 day exposure, the digestion with FPG ($GDI_{FPG}$; Fig. 2A) demonstrated significantly higher damage, in relation to the control, in all treatment groups. $NSS_{FPG}$ (Fig. 2B) value in the lower concentration of Roundup® was significantly higher than the control. Moreover, the group exposed to the lower concentration of the commercial formulation displayed significantly higher levels of oxidative damage than those exposed either to the active ingredient or to the surfactant in equivalent concentrations (1R1 *vs*. 1G1 and 1R1 *vs*. 1P1).

Regarding 3 days exposure and $GDI_{FPG}$ parameter (Fig. 2A), the same pattern of results was observed as described for 1 day exposure. However, $NSS_{FPG}$ levels (Fig. 2B) revealed no significant differences to the control.

No temporal variations were found for both $GDI_{FPG}$ and $NSS_{FPG}$ data.

Table 2 revealed a significant effect of the factors agent and time, as well as a significant interaction agent x concentration, for both $GDI_{FPG}$ and $NSS_{FPG}$. In addition, an interaction agent x time was also found for $NSS_{FPG}$.

*EndoIII associated DNA breaks*

After 1 day exposure, the digestion with EndoIII revealed damage levels ($GDI_{EndoIII}$; Fig. 3A) significantly higher than the control in all treatment groups. As far as the $NSS_{EndoIII}$ parameter (Fig. 3B) is concerned, none of the conditions showed significant differences in relation to the control, despite the clear increase displayed by 1R1.

Considering 3 days exposure, all the treatment groups displayed a significant increase in $GDI_{EndoIII}$ levels *versus* the control group (Fig. 3A). Comparing the effect of both Roundup® ingredients following isolated exposure, fish exposed to the highest POEA concentration demonstrated significantly increased $GDI_{EndoIII}$ values compared to those exposed to the equivalent glyphosate concentration (3P2 *vs*. 3G2). $GDI_{EndoIII}$ concentration-dependent increase was only detected for Roundup®. A time-related decrease was detected in $GDI_{EndoIII}$ for the highest glyphosate concentration (1G2 *vs*. 3G2). Considering $NSS_{EndoIII}$ (Fig. 3B), only the highest concentration of glyphosate showed an increase in relation to the control.

Table 2 revealed a significant effect of agent, concentration and time on $GDI_{EndoIII}$ levels, as well as significant interactions agent x concentration and agent x time. $NSS_{EndoIII}$ showed to be significantly affected by time and displayed also a significant interaction agent x time x concentration.

*FPG plus EndoIII associated DNA breaks*

As described for $GDI_{FPG}$ and $GDI_{EndoIII}$ separately, the parameter $GDI_{FPG+EndoIII}$ (Fig. 4) displayed values significantly higher than the control in all the treatments.

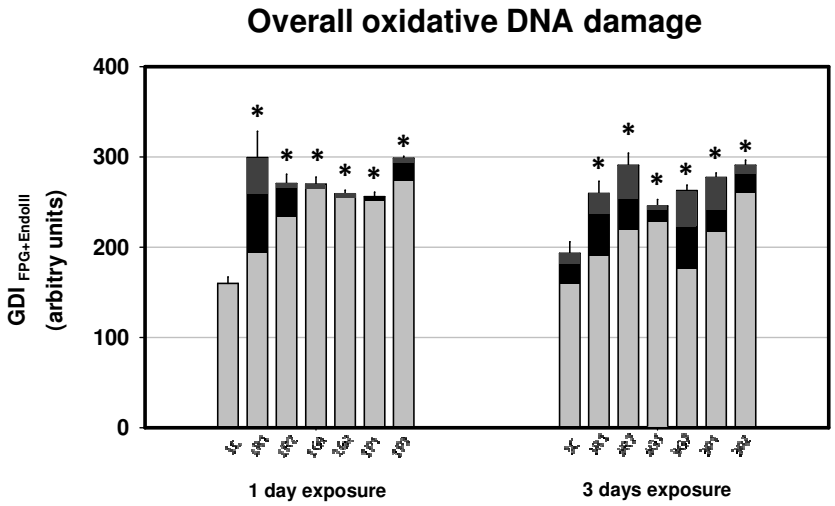


**Fig. 4.** Mean values of DNA damage, measured by comet assay in blood cells of *A. anguilla* exposed to 58 and 116 µg.L$^{-1}$ Roundup® (R1, R2), 17.9 and 35.7 µg.L$^{-1}$ glyphosate (G1, G2) or 9.3 and 18.6 µg.L$^{-1}$ polyethoxylated tallowamine (POEA; P1, P2), during 1 and 3 days (in the abbreviations for test conditions, the first number represents the exposure duration). Values resulted from the sum of genetic damage indicator (GDI; light grey) with additional DNA breaks corresponding to net FPG-sensitive sites ($NSS_{FPG}$; black) and net EndoIII-sensitive sites ($NSS_{EndoIII}$; dark grey). Bars represent the standard error. Statistically significant differences ($p<0.05$) are: (*) in relation to control (C), within the same exposure time.

Moreover, $GDI_{FPG+EndoIII}$ showed a significant effect of agent, as well as a significant interaction agent x time x concentration (Table 2).

### 4.4 Discussion

Taking as a departing point the genotoxicity of Roundup® (at environmentally realistic levels) demonstrated in fish (Guilherme et al. 2010), the major purpose of this work was to improve the knowledge on the DNA damaging potential of its components (glyphosate and POEA) individually. This approach is relevant since, regardless of their jointly agrochemical application, Roundup® components can affect non-target aquatic organisms singly. Moreover, it also allows recognize eventual chemical interactions,

contributing to better understand how the partial effects contribute to the mixture final effect.

The results in terms of non-specific DNA damage, depicted in GDI values, confirmed the genotoxicity of Roundup® (for the highest concentration and both exposure times), but also demonstrated, for the first time in fish, the genotoxic potential of glyphosate and POEA individually. These findings were also corroborated by the sub-total of damaged nucleoids.

In an attempt to compare the effects of the formulation with those of its components, it should be highlighted that Roundup® revealed to be, most of the time, less genotoxic than the active ingredient or the surfactant alone (with the exception of the higher concentrations after 3 days). Furthermore, fish exposed to Roundup® always showed (for both concentrations and exposure times) a level of damage far lower than expected based on the sum of the effects of the separate components, suggesting an antagonistic interaction between glyphosate and POEA. Moreover, the identification of this type of interaction is reinforced by the observation that both components individually, at the lowest concentrations (G1 and P1) and both exposure times, induced a significant DNA damage increase, which was not verified for Roundup®, therefore configuring an antagonism of mutual interference. In accordance, after 1 day exposure, 1G1 group displayed a significantly higher GDI value than 1R1.

In divergence with the current observations, the two available studies addressing the interactions between glyphosate and POEA in aquatic species pointed to another direction (Howe et al. 2004; Frontera et al. 2011). Thus, Howe and co-workers (2004) found that glyphosate has no acute or chronic effects (measured as malformations and gonadal abnormalities) on developing tadpoles, whereas tadpoles reared in environmentally relevant concentrations of Roundup® formulations suffered mortality and developmental abnormalities. In addition, exposure to POEA alone showed toxicity similar to that of Roundup®, which was regarded as an indication that this surfactant contributes most, if not all, to the acute toxicity of the end-use formulations. On the other hand, Frontera and co-workers (2011) stated that POEA acts synergistically with glyphosate on the crayfish *Cherax quadricarinatus* concerning long-term effects on somatic growth parameters. This highlights that the mechanism of action of these compounds on different parameters (and different levels of biological organization) could be entirely different. Besides species, that the occurrence (or not) of chemicals interactions, as well as the type of interaction [e.g.

supra-additive (synergistic) or infra-additive (antagonistic)], can vary, with the endpoint addressed.

Fish exposed to the highest concentration of glyphosate (G2) for 3 days recovered from the damage detected after 1 day exposure. This could be an adaptation to the genotoxic stimulus allowing them to avoid the damage expression as GDI. Therefore, it can be suggested that the influence of the factor time identified for GDI (see Table 2) was markedly determined by the temporal variation above reported for G2 condition. The perception of this temporal influence was probably more obvious due to the absence of interactions agent x time and concentration x time. Accordingly, G2 group displayed a time-related variation on the most frequent damage classes, depicted in a deviation from classes 2 and 3 (after 1 day) to classes 1 and 2 (after 3 days). It is known that DNA strand breaks and alkali labile sites detected by the comet assay represent an early sign of damage (Lee and Steinert 2003), which might be subject to a repair process (Collins 2004). Hence, the time-related disappearance of DNA damage currently observed in G2 group can be explained by the intervention of a DNA repair system and/or by the catabolism of heavily damaged blood cells in the spleen. These processes were previously presented by Saleha Banu et al. (2001) to explain reductions in comet tail-length after 2 and 3 days and a return to control levels after 4 days in blood cells of fish (*Tilapia mosambica*) exposed to an organophosphate pesticide. Moreover, an increased splenic erythrophagia has also been associated with an intense genetic damage in *A. anguilla* (Pacheco and Santos 2002). In the current study, this adaptive/recovery phenomenon depended on the concentration (only occurred for the highest concentration) and was related to a particular genotoxic agent, since it was not observed for POEA. This is in line with the observation that, comparing the respective highest concentrations, the surfactant displayed the most elevated levels of DNA damage among the studied agents (commercial formulation and components). Accordingly, 3P2 group showed GDI levels significantly higher than 3G2. Despite the absence of genotoxic studies comparing the potential of glyphosate and POEA, the present results corroborate the assumption previously presented by Giesy et al. (2000) and Tsui and Chu (2003) that this surfactant is more toxic than the active ingredient.

The results obtained from the comet assay improved with an extra-step with DNA lesion-specific repair enzymes were presented showing either the overall DNA breaks scored after the incubation with endonucleases (Figs. 2A, 3A and 4) or the additional breaks corresponding to net enzyme-sensitive sites (Figs. 2B and 3B). This improves the

possibility to identify a particular damaging action (e.g. oxidative DNA damage) and the respective particularities that can be masked by the overall breaks score.

$GDI_{FPG}$ results demonstrated significantly higher levels of damage for all the treatments in both exposure lengths. This means that the evaluation of the additional breaks resulting from oxidised purines identified also the lower Roundup® concentration as genotoxic (for both exposure lengths), as well as the highest glyphosate concentration after 3 days, which did not occur for GDI parameter, as discussed above. The highest damage extent, measured as $GDI_{FPG}$, was found for the groups exposed to the highest POEA concentration (in both exposure times); however, those $GDI_{FPG}$ levels should be justified mainly by a great elevation of GDI levels, rather than by a increment specifically in the breaks resulting from oxidised purines.

Comparing the effects of the commercial formulation with those of its constituents in terms of $GDI_{FPG}$, it was notable that, in contrast to GDI, Roundup® displayed levels of damage at the same level as glyphosate and POEA, at both concentrations and exposure times. However, like in GDI, $GDI_{FPG}$ values in fish exposed to Roundup® never showed a level of effect close to the sum of the individual chemical effects, which can be regarded as an antagonistic interaction. $NSS_{FPG}$ results revealed that the genotoxic risk strictly associated to DNA oxidation only occurred for the lowest Roundup® concentration, after 1 day (1R1). Moreover, this result reflected a synergistic interaction, corroborated by the significantly higher $NSS_{FPG}$ levels observed in 1R1 in relation to 1G1 and 1P1. Oxidative DNA damage was reduced after 3 days exposure, which is in line with the detection of a significant effect of the factor time on $NSS_{FPG}$ (Table 2).

As described for $GDI_{FPG}$, $GDI_{EndoIII}$ data revealed significantly higher DNA damage for all the treatments in both exposure lengths, when compared with the respective control. Additionally, the maximum $GDI_{EndoIII}$ levels were found for the highest concentration of POEA in both exposure lengths, which is in line with the significantly higher $GDI_{EndoIII}$ values observed for the group 3P2 in relation to 3G2. Nevertheless, these observations cannot be regarded as an indication that POEA has a potential to induce pyrimidines oxidation higher than the other agents, including glyphosate. This statement is corroborated by $NSS_{EndoIII}$ data, which, despite the notably elevated value (statistically insignificant) displayed by the group 1R1, did not reflect any significant differences between treatments. Moreover, the significant $GDI_{EndoIII}$ increase observed for 3G2 group is strictly related to the significant increase in the oxidised pyrimidines levels ($NSS_{EndoIII}$ data). It was also demonstrated that glyphosate oxidative action increased with time, which is corroborated by the influence of this factor. Returning to the analysis of $GDI_{EndoIII}$

results, it should be highlighted that when the additional breaks resulting from oxidised pyrimidines were considered, the highest Roundup® concentration (3 days) appeared to be more genotoxic than the lowest. This calls for attention to $GDI_{EndoIII}$ parameter as the one able to distinguish between concentrations of the tested agents. Despite the detection of significant factor interactions for agent x time and agent x concentration, the isolated influence of the factors agent, concentration and time could still be recognized. Again, $GDI_{EndoIII}$ results suggest an antagonistic interaction between the Roundup® constituents justified by the above presented reason.

Chemical interactions with a subsequent biological manifestation can occur at several levels, such as outside the body or on chemicals uptake, distribution, biotransformation, interaction with target sub-cellular structures, modulation of defense processes and excretion. Nonionic surfactants, such as POEA, were suggested to exert their adverse effects on aquatic organisms through disruption of the respiratory surfaces (Lindgren et al. 1996b). Though not testing POEA, Partearroyo et al. (1991) showed that a wide range of surfactants interfere with gill morphology and cause lysis of gill epithelial cells in fish (Partearroyo et al. 1991). In this direction, despite the evidence that POEA facilitates membrane penetration of glyphosate in mammalian cell cultures (as in plants) (Richard et al. 2005), a POEA action hampering the uptake of glyphosate through gills may be hypothesized as a mechanism underlying the antagonistic interaction perceived in Roundup® exposures. However, the present results do not clarify at what level(s) glyphosate and POEA interact in order to demonstrate the detected antagonism. This is a matter that remains unexplored and deserves more attention in future works.

It was demonstrated that recently developed glyphosate-based formulations lacking POEA were less toxic to amphibians (Howe et al. 2004). Nevertheless, in terms of the contribution of the surfactant to the overall genotoxicity of the commercial formulation, the present results seem to point out a different direction, since the active ingredient also showed to have a genotoxic action. Anyhow, the elevated genotoxic potential displayed by POEA reinforced the idea that the omission in the label of commercial herbicides of its inclusion is inadequate and its collective identification as ‘‘inert’’ can be misleading, as stated by Peixoto (2005) and Renner (2005). Furthermore, a revision of its hazard classification (presently is “of minimal concern”) by the public regulatory agencies is recommended.

### 4.5 Conclusions

The present findings confirmed the genotoxicity of Roundup®, also demonstrating, for the first time in fish, the genotoxic potential of glyphosate (active ingredient) and POEA (surfactant) individually. Moreover, POEA induced the highest extent of DNA damage, when compared to glyphosate as well as to the commercial mixture. Though both components seem to contribute to the overall genotoxicity of the pesticide formulation, the sum of their individual effects was never observed, pointing out an antagonistic interaction between them.

Keeping in mind a reduction of the risk posed by glyphosate-based formulations to non-target organisms as a function of the surfactant selection, the results demonstrated that POEA, though it is far to be considered biologically inert, does not increase the risk associated to the active ingredient.

The genotoxic action strictly related to oxidized purines was only detected for the lowest Roundup® concentration (1 day exposure), pointing out a synergistic action between glyphosate and POEA for this specific type of damage. On the other hand, damage associated to pyrimidines oxidation was only observed for glyphosate (at the highest concentration, after 3 days exposure). Overall, DNA oxidation was not perceived as a dominant mechanism of damage for the assessed genotoxicants.

In evaluating the methodology adopted, the determination of the non-specific damage as GDI (standard comet procedure) showed some limitations relatively to the scoring of the overall damage encompassing oxidatively induced breaks (comet assay with an extra-step involving DNA lesion-specific repair enzymes). The latter approach revealed to represent a value-added towards an effective assessment of genotoxic hazard, limiting the risk of false negative results.

**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.

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# Chapter V

**DNA and chromosomal damage induced in fish (*Anguilla anguilla*) by aminomethylphosphonic acid (AMPA) - the major environmental breakdown product of glyphosate**

# 5 DNA and chromosomal damage induced in fish (*Anguilla anguilla*) by aminomethylphosphonic acid (AMPA) - the major environmental breakdown product of glyphosate

**Abstract**

Most of studies on the effects of pesticides in aquatic organisms are focused on the active ingredients or on the commercial products as a whole. However, the assessment of the direct impact of chemicals that may occur in the environment as breakdown products of the parental compound could be considered even more ecotoxicologically relevant. Glyphosate, an active ingredient of various well-known herbicide preparations, easily reaches the aquatic compartment being naturally degradated into aminomethylphosphonic acid (AMPA). Despite the persistence of AMPA in water systems, to date no studies were performed concerning its effects in aquatic organisms. In this context, the evaluation of the genotoxic hazard posed by AMPA to fish emerges as critical but unexplored issue. Hence, the main goal of the present research was to assess the genotoxicity potential of AMPA in fish, using the comet and erythrocytic nuclear abnormalities (ENA) assays, as reflecting different levels of damage, i.e. DNA and chromosomal damage, respectively. Therefore, these assays were applied to blood cells of *Anguilla anguilla*, following short-term exposures (1 and 3 days) to environmental realistic concentrations of AMPA (11.8 and 23.6 $\mu g.L^{-1}$). In order to better understand the DNA damaging mechanisms, an extra step was added to the standard procedure of comet assay, where nucleoids were incubated with DNA lesion-specific repair enzymes (FPG and EndoIII). After 1 day exposure, the standard comet assay demonstrated the AMPA potential to induce DNA damage for both concentrations. The third day of exposure revealed that only the highest concentration was able to induce DNA damage. Concerning the use of DNA lesion-specific repair enzymes, neither FPG nor EndoIII reflected the capability of AMPA to exert oxidative damage. Furthermore, the ENA results indicated the potential of AMPA to induce chromosomal lesions, despite its later appearance when compared to damage measured as comet assay. Overall, the present findings pointed out the genotoxic hazard of AMPA to fish and, subsequently, the importance of including it in futures studies concerning the risk assessment of glyphosate-based herbicides in the water systems.

**Keywords**: AMPA; glyphosate; genotoxicity; DNA lesion-specific repair enzymes; fish.

## 5.1 Introduction

Most of studies on the effects of pesticides in (non-target) aquatic organisms have been focused on the active ingredients or on the commercial products as a whole. However, the parental compounds present in the formulations may suffer modifications of their chemical structure in the environment, originating products with different toxic properties. Though frequently overlooked, this perspective triggered studies with the transformation products of endosulfan (a broad-spectrum insecticide) (Hoang et al. 2011) and dichlobenil (a broad-spectrum herbicide) (Björklund et al. 2011), pointing out their potential risk to aquatic biota. Therefore, the assessment of the direct impact of chemicals that may occur in the water systems as breakdown products of the former ingredients should be considered ecotoxicologically unavoidable and included in the priorities of both researchers and public authorities.

Glyphosate [N-(phosphono-methyl-glycine)] is the active ingredient of the most widely used non-selective post-emergence herbicides in the world. Formulations containing glyphosate are heavily used in agriculture, urban landscaping and forestry (Landry et al. 2005; Kolpin et al. 2006). Though it can be intentionally applied to control emergent and floating aquatic vegetation, surface runoff following terrestrial uses is known to be the primary way of glyphosate transfer to surface waters. Studies on environmental fate of glyphosate indicated that it tends to strongly bind to organics matrices, like sediment of aquatic systems, and rapidly degrade (Feng et al. 1990). The soil sorption and the degradation of glyphosate exhibit a great variation depending on soil composition and properties (Gimsing et al. 2004), as well as on the factor leachability (Borggaard and Gimsing 2008). Once in the aquatic environment, glyphosate can be naturally converted into sarcosine and aminomethylphosphonic acid (AMPA) (Landry et al. 2005; Al-Rajab et al. 2008). Of these two, AMPA has the highest occurrence in water, showing an environmental mobility and persistence greater than glyphosate (Kolpin et al. 2006), being thus assumed as the major breakdown product (Williams et al. 2000). Its appearance in water (as well as in soils) is due to a microbiologic degradation rather than to a chemical action (Rueppel et al. 1977).

The relative rapid degradation of glyphosate (half-life from 7 to 14 days) in aquatic environments (Giesy et al. 2000) can, apparently, limit the environmental risk associated. However, this is highly questionable due to the appearance of its metabolites, namely AMPA, which has been found in levels ranging 3.6-60 $\mu g.L^{-1}$ (Battaglin et al. 2005; Struger et al. 2008).

Considering the abundance of studies reporting the occurrence of AMPA as a pollutant in the aquatic environment, it would be expected that its effects on organisms have already been more extensively explored. Nevertheless, this is a matter almost completely uncovered, and relatively little is known about the biological activity of this compound (Mañas et al. 2009), making surprising its categorization by some regulatory agencies as "not of toxicological concern" (USEPA 1993).

The analysis of DNA alterations in aquatic organisms have been shown as a highly suitable method for evaluating the environmental genotoxic contamination, allowing the detection of exposure to low concentrations of contaminants, including pesticides, in a wide range of species (Scalon et al. 2010). Hence, and despite the considerable amount of studies addressing glyphosate and Roundup® (a glyphosate-based herbicide) genotoxic potential to fish (Çavas and Könen 2007; Cavalcante et al. 2008; Guilherme et al. 2010, 2012a; 2012b), the risk posed to genome integrity by AMPA remains unknown. To the author's knowledge, no studies were performed concerning the genotoxicity of AMPA in fish as well as in aquatic organisms. The only study carried out in this direction was applied to mammalian models (human cell lines and mice), clearly demonstrating a genotoxic action (Mañas et al. 2009).

Keeping in mind the knowledge gaps identified, the main goal of the present research was to assess the genotoxic potential of AMPA, as the major breakdown product of glyphosate, following short-term exposures (1 and 3 days) to environmentally realistic concentrations (11.8 and 23.6 $\mu g.L^{-1}$). Genotoxic endpoints such as comet and erythrocytic nuclear abnormalities (ENAs) assays were adopted, since they can reflect different levels of genetic damage, i.e. DNA and chromosomal damage, respectively. The comet assay detects DNA strand breaks and alkali labile sites (Lee and Steinert 2003; Andrade et al. 2004), representing an early sign of damage that can be subjected to a repair process. In order to better understand the DNA damaging mechanisms, an extra step was added to the standard procedure of comet assay where nucleoids were incubated with DNA lesion-specific repair enzymes, highlighting specifically oxidised DNA bases. Complementary, the ENA assay, based on the detection of micronuclei and other nuclear anomalies (Pacheco and Santos 1997), signals chromosome breakage (clastogenicity) or loss and mitotic spindle apparatus dysfunction (aneugenicity) (Fenech 2000; Stoiber et al. 2004), which are hardly reparable lesions. Hence, ENA assay displays later and less transient alterations when compared with those detected by the comet assay.

## 5.2 Material and Methods

### 5.2.1 Chemicals

AMPA and all other chemicals were obtained from the Sigma-Aldrich Chemical Company (Spain). DNA lesion-specific repair enzymes, namely formamidopyrimidine DNA glycosylase (FPG) and endonuclease III (Endo III), were purchased from Professor Andrew Collins (University of Oslo; Norway).

### 5.2.2 Test animals and experimental design

European eel (*Anguilla anguilla* L.) specimens with an average weight 0.25±0.02 g (glass eel stage) were captured from Mondego river mouth, Figueira da Foz, 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 physico-chemical conditions: salinity 0, temperature 20±1ºC, pH 7.3±0.2, nitrate 25±0.5 $mg.L^{-1}$, nitrite 0.03±0.01 $mg.L^{-1}$, ammonia 0.1±0.01 $mg.L^{-1}$, dissolved oxygen 8.1±0.5 $mg.L^{-1}$. During this period, fish were daily fed with fish roe.

The experiment was carried out in 1-L aquaria, in a semi-static mode, under the conditions described for the acclimation period. After acclimation, 72 eels were divided into 6 groups, corresponding to three test conditions and two exposures times (3x2). Thus, fish were exposed to 11.8 and 23.6 $\mu g.L^{-1}$ AMPA (groups A1 and A2, respectively). Another group was kept with clean water - 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 concentrations of glyphosate adopted previously by Guilherme and co-workers (2012b) served as a basis to determine the AMPA concentrations currently tested. Taking this as a starting point, the concentration of AMPA was calculated assuming that it results from a glyphosate conversion on a basis of 1:1 mole. Thus, for instance, it was assumed that 17.9 µg of glyphosate correspond to 11.8 µg of AMPA. Stock solution of AMPA was prepared using deionized water just before addition to exposure water. The experiment was carried out using triplicate (n=3) groups of 4 fish for each condition/time (3x4 = 12 fish).

Fish were not fed during experimental period. Fish were sacrificed by cervical transection at the post-opercular region and blood collected from the heart using heparinised capillary tubes. Blood smears were immediately prepared for ENA assay. Two µL of blood were immediately diluted in 1 mL of ice-cold phosphate-buffered saline (PBS) to prepare a cell suspension, which was kept on ice up to further procedure.

5.2.3 Evaluation of genetic damage

5.2.3.1. Comet assay

The conventional alkaline version of the comet assay was performed according to the methodology of Collins (2004) and adapted by Guilherme et al. (2010), with the proper adjustments to assay procedure with extra step of digesting the nucleoids with endonucleases. In order to significantly increase the throughput of the assay, a system of eight gels per slide was adopted, based on a model created by Shaposhnikov et al. (2010) and adapted by Guilherme et al. (2012b). Briefly, 20 µL of cell suspension (previously prepared in PBS) was mixed with 70 µL of 1% low melting point agarose (in PBS). Eight drops of 6 µL were placed onto the precoated slide as two rows of 4 (4 groups of 2 replicates), without coverslips, containing each gel approximately 1,500 cells. The gels were left for ±5 min at 4 ºC in order 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 ºC, for 1 h. After lysis of agarose-embedded cells, slides were washed 3 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 ºC.

Three sets of slides were prepared: two sets were incubated with endonucleases (1) FPG and (2) EndoIII, that convert oxidised purines and pyrimidines into DNA single strand breaks, respectively (Azqueta et al. 2009), and a third set (3) was incubated only with buffer. Hence, 30 µL of each enzyme diluted in buffer were applied in each gel, along with a coverslip, prior to incubation at 37 ºC for 30 min in a humidified atmosphere. Then, slides were 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 $\mu g.L^{-1}$).

Slides with eight gels each, and 50 nucleoids per gel, were observed, using a Leica DMLS fluorescence microscope (400x 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 this formula:

GDI = [(% nucleoids class 0)×0] + [(% nucleoids class 1)×1] + [(% nucleoids class 2)×2] + [(% nucleoids class 3)×3] + [(% nucleoids class 4)×4]

GDI values were expressed as arbitrary units in a scale of 0 to 400 per 100 scored nucleoids (as average value for the 2 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 designated $GDI_{FPG}$ and $GDI_{EndoIII}$, respectively. Besides GDI, the frequency of nucleoids observed in each comet class was also expressed, as recommended by Azqueta et al. (2009). In order to improve the expression of the DNA damage extent (Palus et al. 1999; Çavas and Könen 2007), the sub-total frequency of nucleoids with medium (class 2), high (class 3) and complete (class 4) damaged DNA was also calculated (2+3+4).

5.2.3.2. ENA assay

This assay was carried out in mature peripheral erythrocytes according to the procedure of Pacheco and Santos (1996). 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 1000x 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 (2000) and Ayllon and Garcia-Vazquez (2001). Final results were expressed as the mean value (‰) of the sum for all the lesions observed (K + L + S + N + MN).

5.2.4 Statistical analysis

Statistica 7.0 software was used for statistical analysis. All data were first tested for normality and homogeneity of variance to meet statistical demands. One-way Analyses of Variance (ANOVA), followed by Dunnett test as Post-hoc comparison, was applied to compare treated with control groups, within the same exposure duration. Two-way ANOVA was applied to test the effect of the factors concentration and exposure time on the levels of DNA damage, as well as on the frequency of nuclear abnormalities. In addition, the interactions between factors were also tested. The Tukey test was applied as Post-hoc comparison. In all the analyses, differences between means were considered significant when $p<0.05$ (Zar 1996). 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 (Zar 1996).

## 5.3 Results

### 5.3.1 DNA damage

#### 5.3.1.1. Non-specific DNA damage

Analysing GDI values after 1 day exposure (Fig. 1), both AMPA groups showed significant increases, relative to the control. The 3 days exposure (Fig. 1) revealed that only the higher concentration of AMPA (A2) induced significant DNA damage, in comparison with the control. Neither concentration nor time related significant differences were observed; however, a decrease tendency was displayed by all treatments in relation to time. In line, table 1 (2-way ANOVA) revealed a significant effect of the factor time on GDI values.

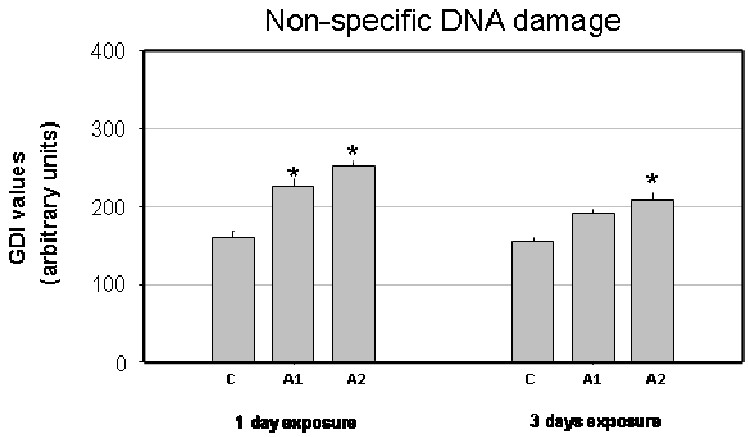


**Fig. 1.** Mean values of genetic damage indicator (GDI) measured by the standard (alkaline) comet assay in blood cells of *A. anguilla* exposed to 11.8 and 23.6 $\mu g.L^{-1}$ aminophosphoric acid (AMPA; A1, A2), during 1 and 3 days. Bars represent the standard error. Statistically significant differences ($p<0.05$) are: (*) in relation to control (C), within the same exposure time.

Considering the results in terms of individual DNA damage classes (Table 2), after 1 day exposure, it was perceptible that only control group (C) displayed a prevalence of classes 1 and 2. Differently, both concentrations of AMPA (A1, A2) presented higher frequencies in classes 2 and 3. Moreover, both treated groups presented significantly lower values concerning class 1 frequency, in relation to control. On the other hand, treated groups showed significantly higher values when class 3 was accounted. Similarly, the sub-total of damaged nucleoids (2+3+4) revealed significantly higher values in both treated groups, in comparison with control.

When 3 days exposure was taken into account (Table 2), the group A1, like the control (C), displayed classes 1 and 2 as the most frequent. In a different way, classes 2

and 3 presented higher frequencies for A2 group. Considering inter-group comparisons within each damage class, A2 group presented significantly lower values than control for class 1, while for class 3 the comparison followed the opposite pattern. The sub-total of damaged nucleoids (2+3+4) displayed significantly higher values only for the group A2, in relation to control.

Comparing 1 and 3 days results displayed in table 2, it was noticeable a general time-related increase in the frequency of class 1, despite the absence of significant differences. In addition, class 3 showed the opposite temporal variation, with a significant decrease in A2 group.

**Table 1.** Results of two-way ANOVA testing the effect of concentration and time, as well as the interactions between them (concentration x time) on the levels of DNA and chromosomal damage in blood cells of *A. anguilla* exposed to aminophosphoric acid (AMPA), during 1 and 3 days. Both F and p values are given for each variable. Non significant differences are signalized as "ns".

| Parameter | Factors | | | | Interaction | |
|---|---|---|---|---|---|---|
| | Concentration | | Time | | Concentration x Time | |
| | *F* | *p* | *F* | *p* | *F* | *p* |
| GDI | 3.83 | ns | 11.34 | 0.0098 | 0.15 | ns |
| $GDI_{FPG}$ | 0.04 | ns | 4.13 | ns | 0.26 | ns |
| $NSS_{FPG}$ | 7.31 | 0.0269 | 9.25 | 0.0160 | 0.01 | ns |
| $GDI_{EndoIII}$ | 7.96 | 0.0224 | 21.70 | 0.0016 | 0.01 | ns |
| $NSS_{EndoIII}$ | 0.04 | ns | 0.01 | ns | 0.56 | ns |
| $GDI_{FPG+EndoIII}$ | 0.09 | ns | 2.63 | ns | 0.03 | ns |
| ENA | 6.48 | 0.0344 | 12.52 | 0.0076 | 5.95 | 0.0405 |

**Table 2.** Mean frequencies (%) of each DNA damage class and sub-total of damaged nucleoids (± standard error), measured by the standard (alkaline) comet assay, in blood cells of *A. anguilla* exposed to 11.8 and 23.6 µg.L$^{-1}$ aminophosphoric acid (AMPA; A1, A2), during 1 and 3 days. Statistically significant differences ($p<0.05$) are: (*) in relation to control (C), within the same exposure time; (♦) between exposure times, within the same treatment.

| | | DNA Damage Classes | | | | | |
|---|---|---|---|---|---|---|---|
| | Exposure Conditions | 0 | 1 | 2 | 3 | 4 | Sub-total (2+3+4) |
| 1 day | C | 5.20±5.20 | 38.11±4.31 | 49.03±3.18 | 7.44±1.59 | 0.22±0.22 | 56.69±3.21 |
| | A1 | 0.00±0.00 | 12.75±3.95 * | 50.58±2.21 | 34.33±4.68 * | 2.33±0.86 | 87.25±3.95 * |
| | A2 | 0.00±0.00 | 2.45±0.79 * | 45.66±3.46 | 49.43±3.18 * | 2.46±0.94 | 97.55±0.79 * |
| 3 days | C | 0.00±0.00 | 49.75±3.10 | 45.58±2.63 | 4.67±1.17 | 0.00±0.00 | 50.25±3.10 |
| | A1 | 0.00±0.00 | 27.42±3.50 | 55.17±2.72 | 16.92±2.49 | 0.50±0.34 | 72.58±3.50 |
| | A2 | 0.00±0.00 | 17.17±4.64 * | 58.00±2.66 | 23.17±3.36 *♦ | 1.67±0.59 | 82.83±4.64 * |

5.3.1.2. Oxidative DNA damage

The detection of oxidized bases was achieved by the analysis of the results of the comet assay with an extra step where nucleoids were incubated with DNA lesion-specific repair enzymes - FPG and EndoIII (Figs. 2 - 4).

*FPG associated damage*

Regarding $GDI_{FPG}$ results, both treatments, and both exposure times, showed significant higher damage, in comparison with the control (Fig. 2A). In a different way, $NSS_{FPG}$ parameter was not capable to distinguish any treatment, in relation to the control (Fig. 2B).

Neither concentration nor time related differences were observed for $GDI_{FPG}$ and $NSS_{FPG}$ (Fig. 2) data, despite the general decrease tendency displayed by all the treatments.

Considering the effect of tested factors (Table 1), it was possible to observe that concentration and time exerted a significant effect in $NSS_{FPG}$ parameter.

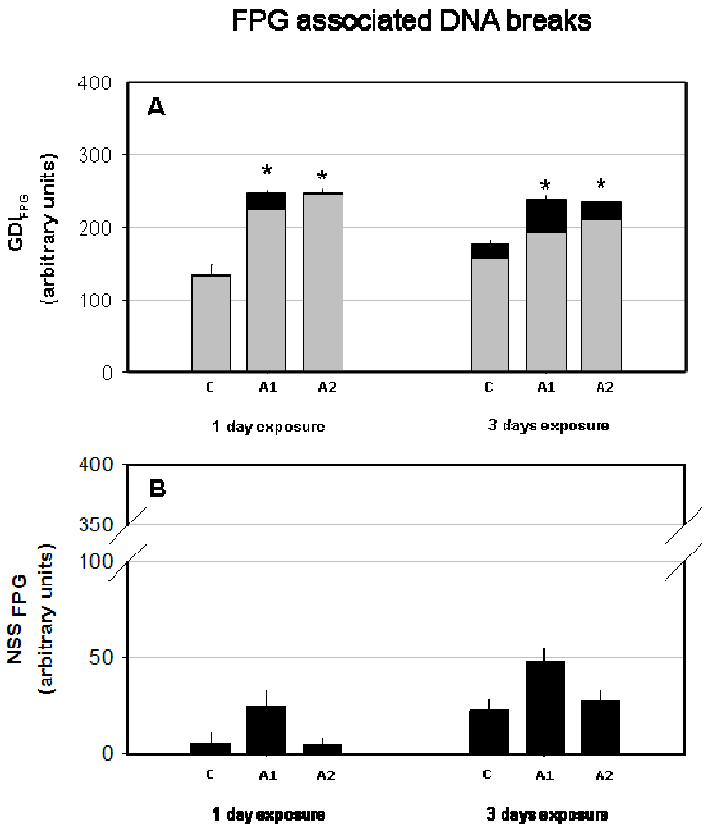


**Fig. 2.** Mean values of DNA damage, measured by comet assay in blood cells of *A. anguilla* exposed to 11.8 and 23.6 µg.L$^{-1}$ aminophosphoric acid (AMPA; A1, A2), during 1 and 3 days. Values resulted from the assay with an extra step of digestion with formamidopyrimidine DNA glycosylase (FPG) to detect oxidised purine bases: (A) overall damage ($GDI_{FPG}$) and partial scores, namely genetic damage indicator (GDI; light grey) 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.

*EndoIII associated damage*

After the 1 day exposure, the digestion with EndoIII ($GDI_{EndoIII}$; Fig. 3A) revealed an overall damage significantly higher than the control, in both treated groups. Concerning the $NSS_{EndoIII}$ parameter (Fig. 3B), no significant differences were found.

Regarding the 3 days exposure, only the group corresponding to the higher concentration of AMPA (A2) showed to be significantly higher than the control ($GDI_{EndoIII}$; Fig. 3A). On the other hand, the $NSS_{EndoIII}$ parameter (Fig. 3B) followed the pattern of the 1 day exposure, being unable to discern any AMPA concentration, when compared with control.

A general time-related decrease was detected in $GDI_{EndoIII}$ values, considering both AMPA groups. Differently, and considering the $NSS_{EndoIII}$ parameter, no differences were found, comparing both exposure times. In addition, table 1 revealed a significant influence of concentration and time factors on the overall damage ($GDI_{EndoIII}$).

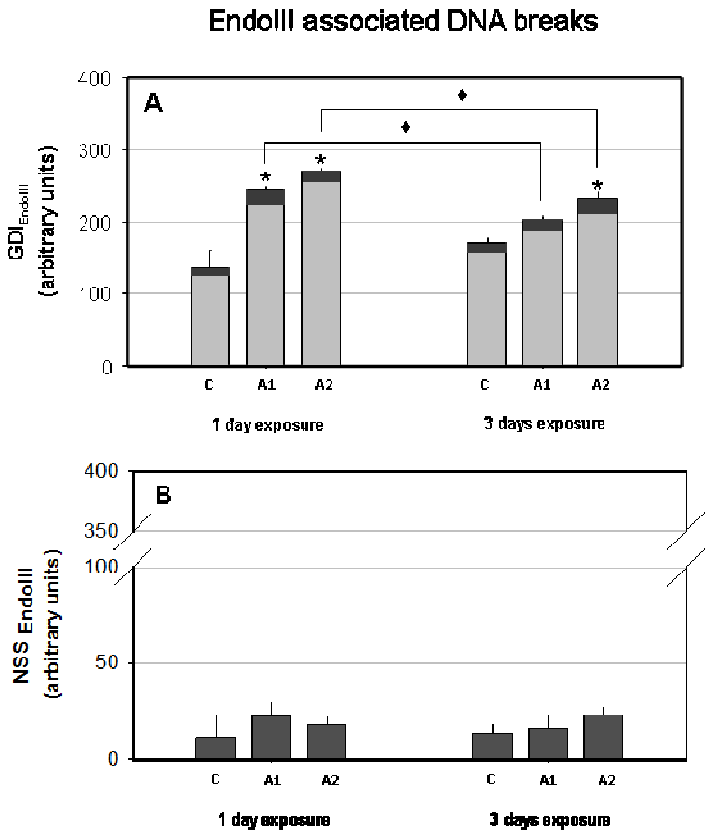


**Fig. 3.** Mean values of DNA damage, measured by comet assay in blood cells of *A. anguilla* exposed to 11.8 and 23.6 µg.$L^{-1}$ aminophosphoric acid (AMPA; A1, A2), during 1 and 3 days. Values resulted from the assay with an extra step of digestion with endonuclease III (EndoIII) to detect oxidised pyrimidine bases: (A) overall damage ($GDI_{EndoIII}$) and partial scores, namely genetic damage indicator (GDI; light grey) and additional DNA breaks corresponding to net EndoIII-sensitive sites ($NSS_{EndoIII}$; dark grey); (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; (♦) between exposure times, within the same treatment.

*FPG plus EndoIII associated DNA breaks*

As described for $GDI_{FPG}$, the parameter $GDI_{FPG+EndoIII}$ (Fig. 4) displayed values significantly higher than the control in both AMPA treatments and both exposure lengths.

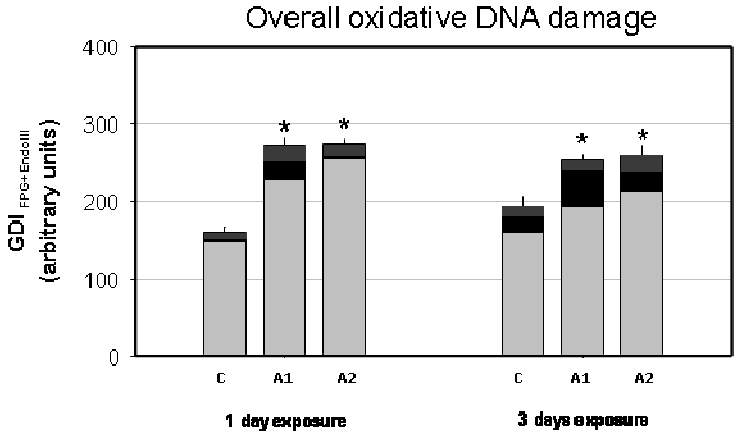


**Fig. 4.** Mean values of DNA damage, measured by comet assay in blood cells of *A. anguilla* exposed to 11.8 and 23.6 µg.$L^{-1}$ aminophosphoric acid (AMPA; A1, A2), during 1 and 3 days. Values resulted from the sum of genetic damage indicator (GDI; light grey) with additional DNA breaks corresponding to net FPG-sensitive sites ($NSS_{FPG}$; black) and net EndoIII-sensitive sites ($NSS_{EndoIII}$; dark grey). Bars represent the standard error. Statistically significant differences ($p<0.05$) are: (*) in relation to control (C), within the same exposure time.

### 5.3.2 Chromosomal damage

No significant alterations were found in ENA frequency following the first day of exposure (Fig. 5). On the other hand, considering the 3 days exposure, it was possible to observe a significant increase for the higher concentration of AMPA (A2), in relation to the control. This exposure condition was the only able to signalize a concentration-dependence, showing a significantly higher chromosomal damage for the higher concentration group (A2) when compared to the lower one (A1) (Fig. 5). Moreover, it was perceptible a time related increase for the higher concentration of AMPA (A2) (Fig. 5).

Table 1 (two-way ANOVA) revealed that ENA parameter was influenced by the factors concentration and time, also showing an interaction between them (concentration x time).

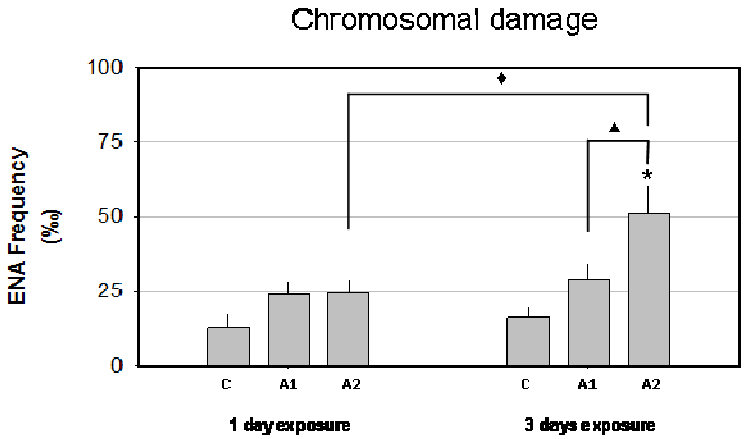


**Fig. 5.** Mean frequency (‰) of erythrocytic nuclear abnormalities (ENA) in blood cells of *A. anguilla* exposed to 11.8 and 23.6 µg.L$^{-1}$ aminophosphoric acid (AMPA; A1, A2), during 1 and 3 days. Bars represent the standard error. Statistically significant differences ($p<0.05$) are: (*) in relation to control (C), within the same exposure time; (▲) between treatments, within the same exposure time; (♦) between exposure times, within the same treatment.

**Table 3.** Mean frequency (‰) of each nuclear abnormality category (± standard error) in peripheral erythrocytes of *A. anguilla* exposed to 11.8 and 23.6 µg.L$^{-1}$ aminophosphoric acid (AMPA; A1, A2), during 1 and 3 days. Statistically significant differences ($p<0.05$) are: (*) in relation to control (C), within the same exposure time; (▲) between treatments, within the same exposure time; (♦) between exposure times, within the same treatment.

| | | Nuclear Abnormalities Categories | | | | | |
|---|---|---|---|---|---|---|---|
| Exposure Conditions | | Kidney Shaped (K) | Lobed (L) | Segmented (S) | Notched (N) | Sub-total (K+L+S+N) | Micronuclei (MN) |
| 1 day | C | 11.63±3.04 | 3.38±0.62 | 4.25±0.97 | 0.00±0.00 | 12.83±4.40 | 0.00±0.00 |
| | A1 | 14.92±2.15 | 5.67±1.59 | 3.58±1.41 | 0.00±0.00 | 24.17±4.29 | 0.00±0.00 |
| | A2 | 13.80±3.05 | 8.40±1.11 | 2.10±0.86 | 0.00±0.00 | 22.09±4.73 | 0.00±0.00 |
| 3 days | C | 9.00±1.66 | 5.67±1.48 | 1.58±0.34 | 0.00±0.00 | 16.25±3.21 | 0.00±0.00 |
| | A1 | 15.08±2.79 | 8.67±1.86 | 5.33±1.94 | 0.00±0.00 | 29.08±5.45 | 0.00±0.00 |
| | A2 | 18.83±2.86 | 23.33±4.97$^{*▲♦}$ | 9.25±2.65$^{*}$ | 0.00±0.00 | 51.42±8.91$^{*▲♦}$ | 0.00±0.00 |

The results in terms of individual analysis of each nuclear lesion category (Table 3) showed no differences in what concerns to 1 day exposure. Contrarily, the 3 days exposure revealed that L and S categories, as well as the sub-total (K+L+S+N), were

significantly higher than the control, when the higher concentration of AMPA (A2) was considered (Table 3). Similarly to what was notice for ENA frequency (Fig. 5), L category and the sub-total displayed significant concentration- and time-related increases (Table 3). Kidney shaped nuclei (K) was the most commonly detected nuclear abnormality in all experiment groups, except in the higher AMPA concentration, after the 3 days exposure (A2) where L category exhibited the highest frequency (Table 3).

### 5.4 Discussion

Despite glyphosate has been considered as practically nontoxic to fish (USEPA 1993), its genotoxic potential to this group of aquatic organisms was recent and unequivocally demonstrated (Guilherme et al. 2012b). The rapid conversion of glyphosate into its breakdown product AMPA appears as a silent problem to the environment, since this metabolite has not been taken into account when the impact of pesticides is under evaluation. The AMPA persistence is higher than glyphosate but its occurrence in the environment has been neglected until now, being its toxicity largely ignored. Consequently, concerns regarding the possible health and environmental hazards emerged, justifying scientific efforts in this direction. Hence, the present study appears as the first study assessing the genotoxic risk of AMPA to fish.

The concentrations of AMPA currently tested were calculated on the basis of environmentally realistic concentrations of glyphosate (Guilherme et al. 2012b), considering its total degradation into the metabolite. Keeping this in mind and the scarcity of data published so far, the following discussion will be mainly focused on the interpretation of the current findings, having as background the available data concerning its precursor – glyphosate.

The genotoxicity of AMPA was assessed by two genotoxic endpoints (comet and ENA), in order to reflect genetic damage at different levels as stated in the introduction. In line with Mañas et al. (2009), the comet assay could be considered a biomarker of genotoxic exposure while ENA assay is a biomarker of genotoxic effect.

In terms of non-specific DNA damage, depicted in GDI values, AMPA showed its genotoxic potential in all tested conditions, except for the lower concentration (A1), after 3 days exposure. Despite statistically non-significant, a time-related decrease tendency in GDI values can be noticed. Taking this together with the lack of a significant GDI increase for the lower concentration (A1) after 3 days, it can be suggested that fish erythrocytes had the capacity of recover from the damage exhibited after 1 day. This idea is confirmed

by the analysis of the DNA damage classes individually, as most of the AMPA treatments displayed classes 2 and 3 as the most prevalent, contrarily to A1 group after 3 days where classes 1 and 2 showed to be predominant. Moreover, class 3 showed a significant decrease from 1 to 3 days exposure. This time-related disappearance of DNA damage concerning A1 groups, which is also reinforced by the significant effect of factor time, can be explained as an adaptation to the genotoxic stimulus allowing fish to avoid the damage expression as GDI. Since it is known that DNA strand breaks and alkali labile sites detected by the comet assay represent an early sign of damage (Lee and Steinert 2003), which might be subject to a repair process (Collins 2004), this recovery may be explained by the intervention of DNA-repair system and/or by the catabolism of heavily damaged cells in the spleen. Additionally, an increased splenic erythrophagia was associated to intense genetic damage in *A. anguilla* (Pacheco and Santos 2002). These processes have been previously presented by Saleha Banu et al. (2001) to explain reductions in comet tail-length after 2 and 3 days and a return to control levels, after 4 days, in blood cells of fish (*Tilapia mosambica*) exposed to an organophosphate pesticide.

The comparison of the DNA damaging effects (measured as GDI) of AMPA presently detected with those of its precursor glyphosate (Guilherme et al. 2012b) reveals that both compounds showed a similar pattern. The only difference noticed is related to the ability of recover from the damage caused. Thus, after 3 days exposure, fish showed to be able to recover from the damage caused by the exposure to 35.7 µg. $L^{-1}$ of glyphosate (the equivalent concentration to the higher concentration of AMPA), while considering the metabolite, fish were only able to recover from the exposure to the lowest concentration (corresponding to 17.9 µg. $L^{-1}$ of glyphosate). Considering the previous facts, it is not possible to sustain that the metabolite (AMPA) is less toxic than the parental compound (glyphosate) as mentioned in a report of the European Commission (2002).

In order to understand a particular damaging action, namely DNA oxidation, the comet assay was improved with an extra-step with two DNA lesion-specific repair enzymes. Thus, the DNA breaks scored after the incubation with endonucleases also pointed out the genotoxicity of AMPA (in all treatments and exposure times, except for $GDI_{EndoIII}$ in A1 group after 3 days exposures). Curiously, when only the additional breaks corresponding to net enzyme-sensitive sites were considered, none of the conditions revealed significant levels of oxidative damage. However, the use of this methodology allows the detection of a genotoxic risk resulting from unspecific (alkali-labile sites, DNA-DNA/DNA-protein cross-linking, and single strand breaks associated with incomplete excision repair sites) and specific (bases oxidation) damage jointly, as well as the isolation

of the oxidative DNA damage. The additional step of the assay also improves the possibility to identify a damaging action that could have been masked by the breaks score as GDI only.

Looking specifically to $GDI_{FPG}$ parameter, after 1 day exposure the results demonstrated to be in accordance with those obtained for GDI. However, when 3 days results as $GDI_{FPG}$ were considered, the lower concentration of AMPA (A1) kept its genotoxic action, pointing out an inability of adaptation to the genotoxic stimulus, contrarily to what has been demonstrated in GDI results. Thus, when DNA damage measured by GDI (alkali-labile sites, DNA-DNA/DNA-protein cross-linking, and single strand breaks associated with incomplete excision repair sites) was complemented with the evaluation of the purine bases oxidation, AMPA confirmed its genotoxicity, revealing also the inability to recover from 1 to 3 days exposure. Accordingly, it can be suggested that the oxidative damage seems to be more difficult to repair, when compared to the non-specific damage. Anyway, it can be inferred that the DNA-repair system played the principal role on the temporal recovery (from 1 to 3 days) displayed by the GDI parameter, rather than the catabolism of heavily damaged cells. The involvement of the latter process would have affected both GDI and $GDI_{FPG}$ parameters (which was not the case).

As described for GDI and $GDI_{FPG}$, $GDI_{EndoIII}$ showed significantly higher DNA damage for both treatments, considering the 1 day exposure. The $GDI_{EndoIII}$ results obtained after the exposure of 3 days, as observed for the GDI parameter and contrarily of $GDI_{FPG}$, did not point out the lower AMPA concentration as being genotoxic.

The $NSS_{EndoIII}$ parameter, similarly to $NSS_{FPG}$, was not able by itself to point AMPA as a notable inducer of oxidative damage. In this way, the potential of AMPA to exert oxidative damage, though it cannot be overlooked, seems to be limited, preventing the detection of damage when only the additional breaks corresponding to the incubation with DNA lesion-specific repair enzymes (FPG and EndoIII) are assessed.

Nevertheless, looking to the $GDI_{FPG+EndIII}$ data (considering the incubation with FPG and EndoIII), the indication towards the genotoxic potential of AMPA was strengthened, since all tested conditions displayed significant DNA damage, which was not observed for the GDI parameter. This pattern of results has been also reported for glyphosate (AMPA precursor) in a previous study carried out with the same species under comparable conditions (Guilherme et al. 2012b). Thus, the present results pointed out a limitation of the standard comet assay (GDI data), as already stated by Guilherme and co-workers (2012b). Likewise, previous results of non-specific DNA damage, depicted by GDI, pointed out the higher concentration of glyphosate (corresponding to the higher

concentration of AMPA currently used) as non genotoxic (Guilherme et al. 2012b). This fact would be disclaimed by results obtained by overall oxidative damage, as well as considering the enzyme-associated DNA breaks (Guilherme et al. 2012b). Contrary to AMPA, the previous study demonstrated that glyphosate was able to induce oxidative damage measured as $NSS_{EndoIII}$ (in a concentration corresponding to the higher AMPA concentration tested). Thus, AMPA showed no evidences to have higher potential to oxidatively damage DNA in comparison with its precursor - glyphosate. On the other hand, an overall evaluation indicates that these two environmental contaminants present similar DNA damaging potential, despite the few differences already mentioned. In spite of the use of different biological models, the present findings on DNA damaging potential of AMPA are in line with those reported by Mañas and co-workers (2009).

Considering the ENA assay, chromosomal damage was only found in fish exposed to the higher concentration of AMPA, after 3 days (A2). In addition, and considering this exposure length, it was possible to distinguish both AMPA concentrations. Moreover, it was also noticed a significant increase of chromosomal damage between different exposure times, when A2 groups were considered. These results were reinforced by the influence of factors concentration and time, as well as by the interaction between them. In what concerns to the individual abnormality categories, the data support the results already discussed. As observed for ENAs frequency, only A2 group after 3 days showed significant differences, either in comparison with corresponding control or A1 group. Moreover, a time related increase was also perceptible. The observed differences were mostly due to the significant increase of the lobed (L) category, despite the slight contribution of S category. In addition, and following the ENAs pattern, L category also displayed a significant increase between both treated groups, during the 3 days exposure, appearing as the only category able to discern between concentrations. A time related increase was also observed for L category (A2 groups), supporting once again its contribution to the total ENAs frequency. The absence of MN in the present study reinforced the usefulness of the other nuclear abnormalities scoring, as previously stated (Guilherme et al. 2008, 2010). The single score of MN may lead to a possible lack of sensitivity related to its low frequency in fish. As mentioned for comet assay, the present ENA results are in line with the findings of Mañas and co-workers (2009) who described an increase frequency of micronucleated erythrocytes in mice, 2 days after an i.p. injection of AMPA.

The comparative analysis of comet and MN (or ENA) assays in terms of their sensitivity is a controversial matter. It is well known that comet assay detects primary DNA

lesions resulting from the balance of DNA damage (strand breaks, alkali-labile sites, and cross-links) and repair mechanisms, while the MN (or ENA) test reveals fixed DNA lesions or irreparable aneugenic effects (Bolognesi et al. 2004). Thus, data resulting from both assays were considered in parallel, as reflecting different types of genetic damage. In this perspective, current ENA data reflected a late appearance of damage when compared to comet assay, as suggested by Wirzinger et al. (2007). This fact seems to be related to the need of the exposed cell population to undergo at least one cell cycle (Udroiu 2006), which is not a requisite for comet assay. Subsequently, only comet assay demonstrated to be able of to detect genetic damage after 1 day of exposure, confirming the precocious nature of the damaging events involved. On the other hand, ENAs, unlike comet assay, demonstrated the ability to distinguish the two tested concentrations. Moreover, it was possible to observe a different pattern related to the temporal evolution of the induced damage. Hence, with the passage of time, the damage magnitude increased concerning the chromosomal damage, while the DNA damage (comet assay) revealed a decrease, pointing out a recovery phenomenon.

In order to clarify the relation between the two endpoints, different correlations were studied (ENAs vs. GDI, r= 0.3363; ENAs vs. GDI $_{FPG+EndoIII}$, r= 0.5743 and ENAs vs. NSS $_{FPG+EndoIII}$, r=0.4883). Accordingly, the total absence of correlations reinforced the idea that genotoxic damage could be caused by different events. Despite ENAs could be originated by DNA single stand breaks (measured by comet assay), the diversity of processes (e.g. DNA repair) involved in this event and the subsequent occurrence of ENAs impairs the existence of correlation between these parameters. Moreover, it cannot be ignored that ENAs could have an aneugenic origin (which can not be measured by comet assay) that can also justify the absence of correlation.

Briefly, it can be inferred that these two genotoxic end points provide complementary information, allowing a more effective assessment of AMPA genotoxic effects, when jointly applied. In this direction, only comet assay detected effects after 1 day exposure, while only ENA assay showed the aptitude to distinguish the tested concentrations and to reflect temporal variations. Accordingly, Wirzinger et al. (2007) stated previously that both are non-specific biomarkers which reflect different forms of environmental stress, recommending the application of both tests.

### 5.5 Conclusions

The present findings demonstrated, for the first time in fish, the genotoxicity of AMPA, expressed both as DNA (comet assay) and chromosomal (ENA assay) damage. Overall, AMPA displayed a genotoxic potential comparable to its precursor (glyphosate), bringing to the fore a recent publication of our research group (Guilherme et al. 2012b).

In an attempt to clarify the mechanisms involved in the detected damaging action, the results indicated that AMPA did not induce a marked DNA oxidation. Nevertheless, the use of DNA lesion-specific repair enzymes as an extra-step to the standard methodology of comet assay appears as a value-added towards an effective assessment of genotoxic hazard.

Finally, it is strongly recommended to include AMPA in futures studies concerning the risk assessment of glyphosate-based herbicides due to its rapid appearance in the water systems and the potential risk to aquatic organisms, namely fish.

**Ethical statement**

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.

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# Chapter VI

**Genotoxicity of the herbicide Garlon® and its active ingredient (triclopyr) in fish (*Anguilla anguilla* L.) using the comet assay**

# 6 Genotoxicity of the herbicide Garlon® and its active ingredient (triclopyr) in fish (*Anguilla anguilla* L.) using the comet assay

**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. Though DNA is frequently regarded as a target of pesticides 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 standard procedure and with an extra step involving DNA lesion-specific repair enzymes) was used to identify DNA damage in blood cells of *Anguilla anguilla* L., following short-term exposures (1 and 3 days) to the previous agents, adopting environmentally realistic concentrations (67.6 and 270.5 $\mu g.L^{-1}$ Garlon®, 30 and 120 $\mu g.L^{-1}$ triclopyr). The results concerning the non-specific DNA damage proved the risk of the herbicide Garlon® and its active ingredient triclopyr, in both tested concentrations and exposure lengths. In addition, it was demonstrated the higher genotoxic potential of the formulation, in comparison to the active ingredient. 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 recommend the adoption of mitigation measures related to the application of these agrochemicals in the framework of forestry and agriculture sustainable management.

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

## 6.1 Introduction

The proper use of herbicides, affecting only restricted terrestrial areas, is a critical issue on forestry and agriculture sustainable management. Nevertheless, following broadcast applications, from the ground or aircraft, these agrochemicals frequently reach the aquatic environment by runoff, 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).

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 to 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 (TEA). However, there are substantial differences in toxicity of TBEE and TEA derivatives, with the former showing to be more toxic in aquatic settings (MMWD 2008).

In natural environments, TBEE is degradated 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 and co-workers (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) and by the detection of avoidance behaviour and growth impairment in amphibians (Wojtaszek et al. 2005). However, only a few sub-lethal parameters concerning Garlon® adverse effects have been assessed in fish. In this context, Kreutzweiser and co-workers (1995) reported a growth inhibition in rainbow trout exposed to Garlon®, while 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 to their commercial products, it becomes particularly relevant to point out the information depicted in the product label (Dow Agrosciences), classifying Garlon® as highly toxicity to fish. Thus, it appears evident the importance to evaluate parameters that can better predict the fish condition following exposure to Garlon®, as suggested by a Canadian health department (Health Canada 1991). Additionally, to the authors' knowledge, no studies were performed concerning the toxicity to fish of the active ingredient of Garlon®, individually.

DNA is a frequent target of pesticides toxicity. In line with this, 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 the possible genetic damage, remain completely unknown.

Bearing in mind the knowledge gaps above identified, the main goal of the present research was to assess the genotoxic potential of the herbicide Garlon® and the active ingredient triclopyr in fish. Therefore, the comet assay was used to identify DNA damage in blood cells of *Anguilla anguilla* L., following a short-term exposure to the previous agents, 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. This combined methodology allows the detection of a genotoxic risk resulting from unspecific and specific damage. Hence, this additional step intended to clarify if the induced damage has an oxidative cause.

## 6.2 Material and Methods

### 6.2.1 Chemicals

The experiment was conducted using the commercial formulation Garlon®, distributed by Dow AgroSciences (Lusosem, Portugal), containing triclopyr formulated as a butoxyethyl ester 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 formamidopyrimidine DNA glycosylase (FPG) and endonuclease III (EndoIII) were purchased from Professor Andrew Collins (University of Oslo, Norway). All the other

chemicals needed to perform comet assay were obtained from the Sigma-Aldrich Chemical Company (Spain).

### 6.2.2 Test animals and experimental design

European eel (*Anguilla anguilla* L.) specimens with an average weight 0.25±0.02 g (glass eel stage) were captured from Mondego river mouth, Figueira da Foz, 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 physico-chemical conditions: salinity 0, temperature 20±1ºC, pH 7.1±0.1, nitrate 25±0.4 $mg.L^{-1}$, nitrite 0.04±0.03 $mg.L^{-1}$, ammonia 0.2±0.03 $mg.L^{-1}$, 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 semi-static mode, under the conditions described for the acclimation period. After acclimation, 120 eels were divided into ten groups, corresponding to five test conditions and two exposures times (5x2). Thus, fish were exposed to 67.6 and 270.5 $\mu g.L^{-1}$ Garlon® (groups G1 and G2, respectively) and 30 and 120 $\mu g.L^{-1}$ triclopyr (groups T1 and T2, respectively). Another group was kept with clean water - 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 deionised 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 4 fish for each condition/time (3x4 = 12 fish). Fish were not fed during experimental period.

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

6.2.3 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), in order to increase the assay output. Briefly, 20 µL of cell suspension (previously prepared in PBS) were mixed with 70 µL of 1% low melting point agarose, in distilled water. Eight drops of 6 µL were placed onto the precoated slide as two rows of 4 (4 groups of 2 replicates), without coverslips, containing each gel approximately 1500 cells. The gels were left for ±5 min at 4 ºC in order 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 ºC, for 1 h. After lysis of agarose-embedded cells, slides were washed 3 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 ºC.

Three sets of slides were prepared: two sets were incubated with endonucleases (1) FPG and (2) EndoIII, that convert oxidised 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) were applied in each gel, along with a coverslip, prior to incubation at 37 ºC 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 $\mu g.L^{-1}$).

Fifty nucleoids were observed per gel, using a Leica DMLS fluorescence microscope (400x 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 this formula:

GDI = [(% nucleoids class 0)×0] + [(% nucleoids class 1)×1] + [(% nucleoids class 2)×2] + [(% nucleoids class 3)×3] + [(% nucleoids class 4)×4]

GDI values were expressed as arbitrary units in a scale of 0 to 400 per 100 scored nucleoids (as average value for the 2 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 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. In order to better estimate the overall magnitude of oxidative DNA damage, the sum of GDI with additional DNA breaks corresponding to both net FPG- ($NSS_{FPG}$) and EndoIII-sensitive sites ($NSS_{EndoIII}$) was also calculated ($GDI_{FPG+EndoIII}$). Moreover, the frequency of nucleoids observed in each comet class considering $GDI_{FPG}$ and $GDI_{EndoIII}$ was also expressed, as recommended by Azqueta et al. (2009).

### 6.2.4 Statistical analysis

Statistica 7.0 software was used for statistical analysis. All data were first tested for normality and homogeneity of variance to meet statistical demands. One-way Analysis of Variance (ANOVA), followed by Dunnett 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 between them. The Tukey test was applied as Post-hoc comparison. In all the analyses, differences between means were considered significant when $p<0.05$ (Zar 1996).

## 6.3 Results

### 6.3.1 Non-specific 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) comparing with the equivalent concentration of its active ingredient (triclopyr) (3T2) (Fig. 1). No time related differences were detected.

Table 1 (3-way ANOVA results) revealed a significant effect of the factors agent, concentration and time on GDI levels, as well as significant interactions agent x concentration and agent x time.

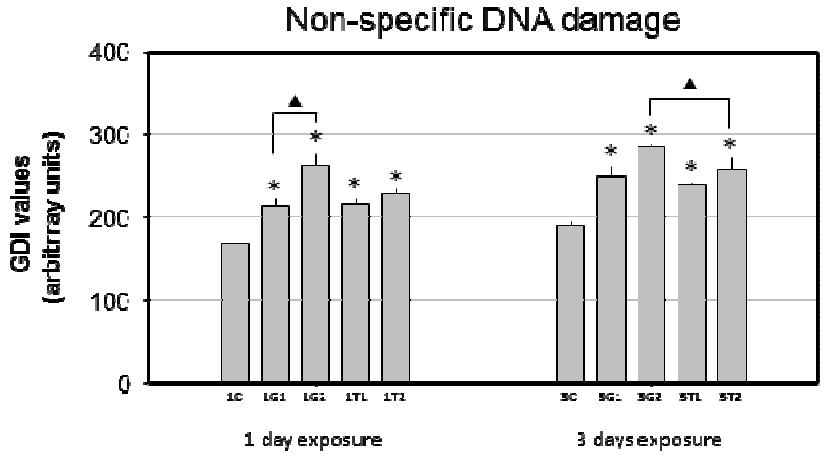


**Fig 1.** Mean values of genetic damage indicator (GDI) measured by the standard (alkaline) comet assay in blood cells of *A. anguilla* exposed to 67.6 and 270.5 µg.L$^{-1}$ Garlon® (G1, G2) and 30 and 120 µg.L$^{-1}$ triclopyr (T1, T2), during 1 and 3 days (in the abbreviations for test conditions, 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; (▲) between treatments, within the same exposure time.

### 6.3.2 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).

*FPG associated damage*

After 1 day exposure, the digestion with FPG revealed damage levels ($GDI_{FPG}$; Fig. 2A) significantly higher than the control in all treated groups. Moreover, the higher concentration of active ingredient (1T2) displayed significantly lower damage when compared to 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. 2B) is concerned, none of the conditions showed significant differences in relation to the control.

Concerning 3 days exposure (Fig. 2A), all treated groups displayed values significantly higher than control. In line with 1 day observations, $NSS_{FPG}$ parameter (Fig. 2B) did not showed any significant difference.

A time-related increase was found concerning the higher concentration of triclopyr (T2) (Fig. 2A).

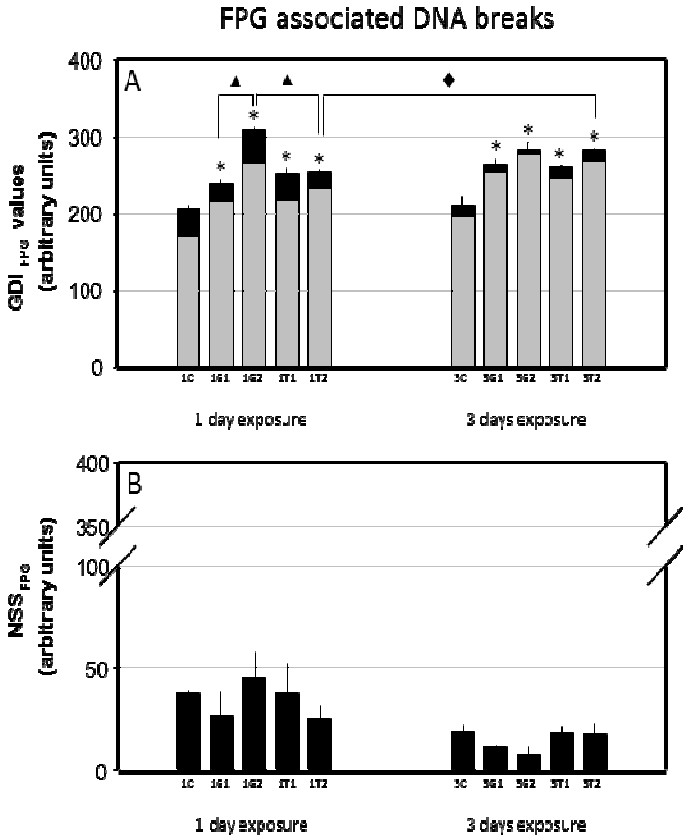


**Fig 2.** Mean values of DNA damage, measured by comet assay in blood cells of *A. anguilla* exposed to 67.6 and 270.5 µg.L$^{-1}$ Garlon® (G1, G2) and 30 and 120 µg.L$^{-1}$ triclopyr (T1, T2), during 1 and 3 days (in the abbreviations for test conditions, the first number represents the exposure duration). Values resulted from the assay with an extra step of digestion with formamidopyrimidine DNA glycosylase (FPG) to detect oxidised purine bases: (A) overall damage ($GDI_{FPG}$) and partial scores, namely genetic damage indicator (GDI; grey) 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; (♦) between exposure times, within the same treatment.

Table 1 revealed, for $GDI_{FPG}$, a significant effect of agent, concentration and time, as well as significant interactions agent x time and agent x time x concentration. $NSS_{FPG}$ showed only to be significantly affected by time.

**Table 1.** Results of three-way ANOVA testing the effect of agent, concentration and time, as well as the interactions between them (agent x concentration, agent x time, concentration x time and agent x time x concentration) on the levels of DNA damage in blood cells of *A. anguilla* exposed to Garlon® or triclopyr, during 1 and 3 days. Both F and p values are given for each variable. Non significant differences are signalized as "ns".

| Parameter | Factors | | | | | | Interactions | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Agent | | Concentration | | Time | | Agent x Concentration | | Agent x Time | | Concentration x Time | | Agent x Time x Concentration | |
| | *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 |
| **$GDI_{FPG+EndoIII}$** | 4.22 | ns | 9.97 | <0.05 | 3.05 | ns | 7.88 | <0.05 | 0.55 | ns | 2.07 | ns | 0.78 | ns |

In order to better understand the behaviour of the DNA damage depending on the tested agent and exposure length, the damage classes were analyzed individually, considering the $GDI_{FPG}$ parameter (Table 2). 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, while triclopyr groups exhibited a prevalence of class 3. Garlon® groups, in particular, presented class 2 and 3 as the most representatives. For class 4, a significant frequency increase observed was only evident for groups corresponding to the highest concentrations of Garlon® (1 and 3 days) and triclopyr (3 days).

**Table 2.** 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, G2) or 30 and 120 µg.$L^{-1}$ triclopyr (T1, T2), during 1 and 3 days (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; (♦) between exposure times, within the same treatment.

| 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±1.61b |
| 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* |

*EndoIII associated damage*

In what concerns to the $GDI_{EndoIII}$ parameter (Fig. 3A), 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 to the correspondent concentration of the commercial formulation (1G2). This parameter, as described for GDI and $GDI_{FPG}$, was able to show a concentration-dependent increase for Garlon® groups (1G1 and 1G2) (Fig. 3A). $NSS_{EndoIII}$ revealed no significant differences in any of the exposure times (Fig. 3B).

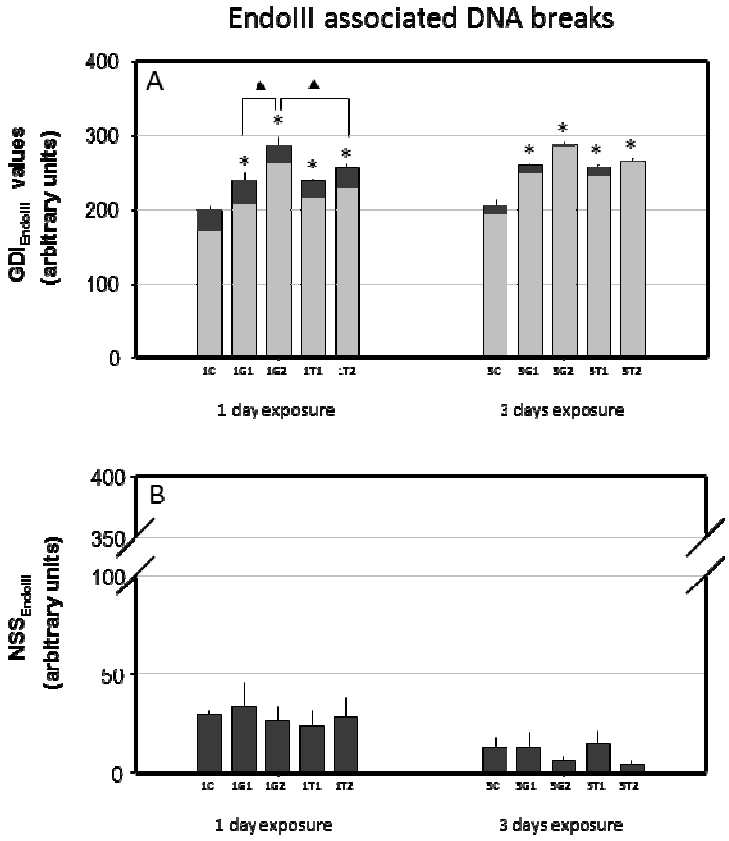


**Fig 3.** Mean values of DNA damage, measured by comet assay in blood cells of *A. anguilla* exposed to 67.6 and 270.5 µg.$L^{-1}$ Garlon® (G1, G2) and 30 and 120 µg.$L^{-1}$ triclopyr (T1, T2), during 1 and 3 days (in the abbreviations for test conditions, the first number represents the exposure duration).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

Table 1 displayed significant effects of agent, concentration and time, as well as interaction agent x concentration were depicted in 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 3). Control groups (at both exposure times) displayed class 2 as the most frequent. Treated groups 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).

**Table 3.** 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, G2) or 30 and 120 µg.L$^{-1}$ triclopyr (T1, T2), during 1 and 3 days (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; (♦) between exposure times, within the same treatment.

| 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 |

*FPG plus EndoIII associated DNA breaks*

As described for $GDI_{FPG}$ and $GDI_{EndoIII}$ separately, the parameter $GDI_{FPG+EndoIII}$ (Fig. 4) displayed values significantly higher than the control in all the treatments (for both 1 and 3 days of exposure) with the unique exception for the group 1G1. Moreover, it was confirmed the significant difference between both Garlon® groups (1G1 and 1G2) after 1 day exposure. In addition, $GDI_{FPG+EndoIII}$ showed a significant effect of concentration, as well as a significant interaction agent x concentration (Table 1).

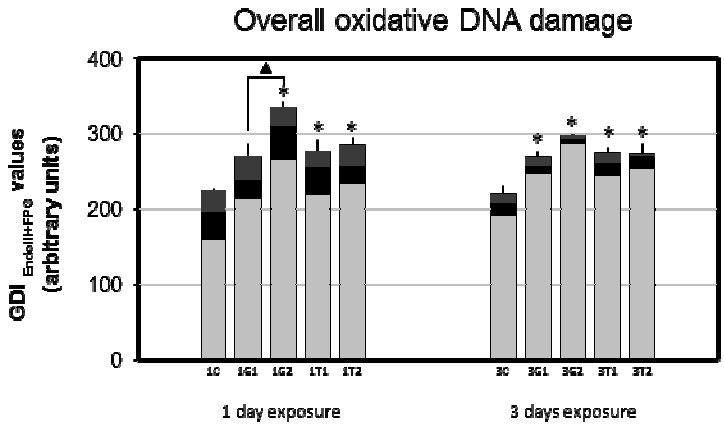


**Fig 4.** Mean values of DNA damage, measured by comet assay in blood cells of *A. anguilla* exposed to 67.6 and 270.5 µg.$L^{-1}$ Garlon® (G1, G2) and 30 and 120 µg.$L^{-1}$ triclopyr (T1, T2), during 1 and 3 days (in the abbreviations for test conditions, the first number represents the exposure duration). Values resulted from the sum of genetic damage indicator (GDI; light grey) with additional DNA breaks corresponding to net FPG-sensitive sites ($NSS_{FPG}$; black) and net EndoIII-sensitive sites ($NSS_{EndoIII}$; dark grey). 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.

### 6.4 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.

Besides the complete absence of genotoxicity evaluation on fish previously highlighted, there is also a scarcity of studies addressing other sub-lethal effects of Garlon® and triclopyr, despite their recognized hazard to aquatic environment. Hence, Kreutzweiser and co-workers (1995) assumed the toxicity of TBEE to fish, but, to be precise, they assessed the toxicity of the commercial product Garlon 4® as a whole. Since 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; Kreutzweiser et al. 1995) adopted concentrations excessively high (0.25 – 7.6 $mg.L^{-1}$) (Janz et al. 1991; Kreutzweiser et al. 1995), comparing to those that commonly are found in the environment. Therefore, the authors consider 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 non-specific DNA damage, the current results expressed as GDI proved the genotoxicity of Garlon® and triclopyr in both tested concentrations and both exposure lengths. It was also perceptible a dose-dependence of the detected potential for the commercial formulation, in particular after the first day of exposure.

On the other hand, the significant difference between the genotoxic potential of both tested agents (considering the highest concentration, after the third day of exposure) call the attention towards the higher toxicity 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). This fact underlines the highest hazard of the commercial formulation, in comparison with the active ingredient.

The value-added concerning the use of DNA lesion-specific repair enzymes as an extra-step to the standard methodology of comet assay in the assessment of the pesticides genotoxicity has already been demonstrated (Guilherme et al. 2012). This improved methodology allows the jointly detection of the genotoxic risk concerning bases oxidation and the non-specific damage (alkali-labile sites, DNA-DNA/DNA-protein cross-linking, and single strand breaks associated with incomplete excision repair sites). In addition, this approach also enables the isolation of the oxidative DNA damage. Thus,

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). Both $GDI_{FPG}$ and $GDI_{EndoIII}$ parameters pointed out the concentration-dependence of Garlon® effects, after 1 day, as reported for GDI. Similarly, these parameters reinforced the GDI indication towards the higher genotoxic potential of Garlon® in relation to triclopyr. $GDI_{FPG}$, in particular, also revealed an increased triclopyr genotoxicity over time, thereby reflecting an augmented risk related with its persistence in the aquatic environment.

The analysis of the individual damage classes (concerning both $GDI_{FPG}$ and $GDI_{EndoIII}$ parameters) revealed that either the concentration or the exposure time exerted influence in the magnitude of damage, since higher concentrations and longer time periods presented higher frequencies in class 3. The notorious prevalence of class 3 in Garlon® groups emphasizes the hazard caused by this herbicide. Moreover, it can be noticed that along the experiment almost all nucleoids presented damage corresponding to classes 2, 3 and 4, reflecting a time-related risk increased.

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. However, looking specifically to these results, it can be noticed that after 1 day exposure higher values were recorded comparing to the third day, despite the absence of any significance. This fact is supported by the significant effect of factor time, for both parameters. Keeping this in mind, the oxidative potential of both tested agents appears to be limited, even though it should not be neglected.

Accordingly, the present findings allowed proving the risk of the herbicide Garlon® to non-target organisms, highlighting, at the same time, its higher genotoxicity in comparison to its active ingredient triclopyr.

Considering that the formulation Garlon® has kerosene (as adjuvant) among its constituents, the toxicity of the latter should be taken into account when the whole formulation is under evaluation due to its recognized toxicity (Arif et al. 1997). However, a USFS (United States Forest Service) report states that the toxicity of kerosene to aquatic species is approximately 100–1,000 folds less than TBEE, suggesting that the acute aquatic toxicity of Garlon® is dominated by TBEE (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 appear to contribute to the product's toxicity. Nevertheless, the interaction between constituents inside the mixture should not be neglected, since Lohani et al. (2000) found that kerosene can elevate the genotoxic

potential of another agent. Keeping this in mind, can Release® (a triclopyr-based herbicide similar to Garlon® without kerosene) be a better choice in what concerns to this kind of herbicides?

Guilherme et al. (2012) found that the active ingredient glyphosate may be more genotoxic than the corresponding commercial formulation (Roundup®) indicating, in this particular case, that the surfactant did not contributed to the mixture genotoxicity.

Several studies with pesticides (and its constituents and/or metabolites) revealed their genotoxic potential through the use of the standard comet assay (Çavas and Könen 2007; Sharma et al. 2007; Guilherme et al. 2010) (Guilherme et al., submitted). These facts pointed out the subsequent hazard of this kind of contaminants to the aquatic environment, even when low concentrations were considered. Moreover, the comet assay has already proved to be highly suitable as tool for the evaluation of exposure to environmental realistic concentrations of herbicides.

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 **(**ERA**)** of pesticides contaminated sites.

### 6.5 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, Garlon® seemed to be more genotoxic than triclopyr.

The ability in exert 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 $NSS_{EndoIII}$). Though the oxidative potential of both tested agents appeared to be limited, it should not be completed neglected.

Thus, the present findings on genotoxic properties of the assessed agents call the attention to the hazard to non-target organisms, namely fish, exposed to these agrochemicals, even when low levels are considered. Consequently, the application of such herbicides should be reconsidered in order to include mitigation measures, in the framework of forestry and agriculture management.

**Ethical statement**

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.

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# Chapter VII

**Are DNA-damaging effects induced by herbicide formulations (Roundup® and Garlon®) in fish transient and reversible upon cessation of exposure?**

# 7 Are DNA-damaging effects induced by herbicide formulations (Roundup® and Garlon®) in fish transient and reversible upon cessation of exposure?

**Abstract**

Owing to the crops seasonality and subsequent periodic/seasonal application of herbicides, their input to the aquatic systems is typically intermittent. Consequently, fish exposure to this type of contaminants can be short and followed by a period of permanence in non-contaminated areas. Bearing this in mind, the assessment of genotoxic endpoints in fish after removal of the contamination source appears as a crucial step to improve the knowledge on the dynamics of herbicide induced genotoxicity, as well as to determine the actual magnitude of risk posed by these agrochemicals to fish. Therefore, the present study intended to shed light on fish ability to recover from the DNA damage induced by short-term exposures to the herbicide formulations Roundup® (glyphosate-based) and Garlon® (triclopyr-based) upon the exposure cessation. European eel (*Anguilla anguilla*) was exposed to the previous commercial formulations, for 3 days, and allowed to recover for 1, 7 and 14 days (post-exposure period). The comet assay was used to identify the DNA damage in blood cells during both exposure and post-exposure periods. As an attempt to clarify the DNA damaging mechanisms involved, an extra-step including the incubation of the nucleotides with DNA lesion-specific repair enzyme was added to the standard comet. The genotoxic potential of both herbicides was confirmed, concerning the exposure period. In addition, the involvement of oxidative DNA damage on the action of Roundup® (pointed out as pyrimidine bases oxidation) was demonstrated, while for Garlon® this damaging mechanism was less evident. Fish exposed to Garlon®, though presenting some evidences towards a recovery tendency, didn't achieve a complete restoration of DNA integrity. In what concerns to Roundup®, a recovery was evident when considering non-specific DNA damage, on day 14 post-exposure. In addition, this herbicide was able to induce a late oxidative DNA damage (day 14). It was also recognized that blood cells of *A. anguilla* exposed to Roundup® were more successful in repairing damage with a non-specific cause, than that associated to bases oxidation. Overall, the present findings highlighted the genetic hazard to fish associated to the addressed agrochemicals, reinforcing the hypothesis of long-lasting damage.

**Keywords**: Garlon®; Roundup®; genotoxicity; recovery; fish.

## 7.1 Introduction

Aquatic contamination became a huge environmental concern since the aquatic compartment was converted into a receptacle of several undesirable contaminants, namely agrochemicals. In this context, herbicides, widely used to control weeds in forestry and agriculture, can reach the aquatic systems by uncontrolled runoff, aerial drift, or inadvertent overspray, affecting fish in particular.

Owing to the crops seasonality and subsequent periodic/seasonal application of these weed-killers, their input to the aquatic systems is typically intermittent. For this reason, as well as due to fish behaviour (e.g. avoidance responses) and mobility (e.g. feeding, refuge-seeking or spawning migrations), their exposure to this type of contaminants can be short (time-scale of days) and followed by a period of permanence in non-contaminated areas. Therefore, the assessment of toxicity endpoints in fish after removal of the contamination source, seeking for an eventual recovery from damage previously induced, appears as a crucial step to improve the knowledge on the dynamics of herbicide induced toxicity, as well as to determine the actual magnitude of risk posed by these agrochemicals to fish. Although several ecotoxicological studies have been performed addressing the effects exerted by herbicides in fish (Crestani et al. 2007; Moraes et al. 2007; da Fonseca et al. 2008), the evaluation of the same effects in the post-exposure period is an almost uncovered issue.

Due to DNA role as repository of genetic information, its integrity and stability is a critical factors to life. Therefore, the evaluation of herbicides as potential exogenous sources of DNA damage to fish is a matter that deserves scientific efforts towards a deepen knowledge on the risks associated to these contaminants. Moreover, the perspective previously presented, related to the (hypothetical) transient or reversible nature of the effects, applied to herbicide-induced DNA lesions is completely absent in the literature.

As defined for the majority of toxic effects, genotoxicity can be determined by a cascade of events, beginning with exposure and culminating with the expression of one or more endpoints. This cascade includes genotoxicant absorption, distribution, metabolism (both detoxification and activation), distribution of metabolites, and interaction with nucleic acids (DNA and RNA), damage repair and, finally, excretion (Hodgson 2012). Hence, the progression of genetic lesions in a post-exposure period is determined by the combined interference of the mentioned processes, with DNA repair playing a central role. As a general knowledge, it is recognized that genetic damage could be originated by DNA adducts formation, DNA/DNA, DNA/protein cross-links as well as DNA single/double

strand-breaks (e.g. as a consequence of base oxidation) and their repair. Therefore, DNA repair processes, though essential for cell viability, can play an ambivalent role, diminishing the impact of the genotoxicant on one hand and, on the other, unintentionally amplifying that impact. Several mechanisms of repair can be considered. The reversal of damage is the most direct repair mechanism and the most efficient. When the damage is more severe, the repair required may presume the excision of a base (base excision repair - BER) or even a nucleotide (nucleotide excision repair - NER) (Costa and Eaton 2006). In addition, the mismatch repair (MMR) usually occurs as a result of a replication error in which DNA polymerase incorporates the wrong base (Costa and Eaton 2006).

Popular herbicide formulations, such as Roundup® and Garlon®, are widely used to for site preparation and conifer release in forestry (Relyea 2005; Wojtaszek et al. 2005). Although used with similar purposes, these herbicides belong to different chemical classes. Roundup® is an organophosphate glyphosate-based herbicide, while Garlon® has triclopyr acid as its active ingredient, corresponding to the class of pyridinecarboxylic acids. Despite the need of more studies in order to clarify the processes involved in their damaging action, the genotoxic potential of Roundup® and Garlon® was already demonstrated in fish, following short-term exposures (Çavas and Könen 2007; Cavalcante et al. 2008; Guilherme et al. 2012b) (Guilherme et al. submitted).

Taking as departing point the genotoxic potential of Roundup® and Garlon®, considering environmentally realistic concentrations, the present study intended to shed a light on fish ability to recover from the DNA damage exerted by short-term exposures to these herbicide formulations upon the exposure cessation, thereby contributing to a realistic perspective of the risk assessment. Hence, European eel (*Anguilla anguilla* L.) was exposed to the previously mentioned commercial formulations, for 3 days, and allowed to recover in clean water for 1, 7 and 14 days (post-exposure period). The comet assay was used to identify the DNA damage in blood cells at each moment, during the exposure and post-exposure periods. 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. This methodology allows the detection of a genotoxic risk resulting from unspecific (alkali-labile sites, DNA-DNA/DNA–protein cross-linking, and single strand breaks associated with incomplete excision repair sites) and specific (bases oxidation) damage. Hence, this additional step intent to clarify if the induced damage has an oxidative cause, as well as, in case of an eventual recovery, to relate the recovery rates with the damage kind.

## 7.2 Material and Methods

### 7.2.1 Chemicals

The experiment was conducted using two commercial formulations: 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 POEA (16 %) as surfactant, and Garlon®, distributed by Dow AgroSciences (Lusosem, Portugal), containing triclopyr formulated as a butoxyethyl ester, at a concentration of 480 $g.L^{-1}$ (or 44.4%) as well as kerosene (petroleum distillate) as an adjuvant. DNA lesion-specific repair enzymes, namely formamidopyrimidine DNA glycosylase (FPG) and endonuclease III (EndoIII) were purchased from Professor Andrew Collins (University of Oslo, Norway). All the other chemicals needed to perform comet assay were obtained from the Sigma-Aldrich Chemical Company (Spain).

### 7.2.2 Test animals and experimental design

European eel (*Anguilla anguilla* L.) specimens with an average weight 0.25±0.02 g (glass eel stage) were captured from Mondego river mouth, Figueira da Foz, 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 physico-chemical conditions: salinity 0, temperature 20±1ºC, pH 7.1±0.3, nitrate 27±0.2 $mg.L^{-1}$, nitrite 0.07±0.02 $mg.L^{-1}$, ammonia 0.2±0.04 $mg.L^{-1}$, dissolved oxygen 8.2±0.3 $mg.L^{-1}$. During this period, fish were daily fed with fish roe. The experiments were carried out in 1-L aquaria, in a semi-static mode, under the conditions described for the acclimation period. After acclimation, 96 eels were divided into 8 groups, corresponding to two test conditions. Thus, fish were exposed to 270.5 $\mu g.L^{-1}$ Garlon® (G group) and another group was kept in clean water - control (C group). For each test condition, a 3 days exposure was carried out. After this period, exposed eels were transferred to clean water and sampled at 1, 7 and 14 days after the end of exposure (post-exposure period). The experiment was carried out using triplicate (n=3) groups of 4 fish for each condition/time (3x4 = 12 fish). Water medium in both exposure and post-exposure periods was daily renewed (100%).

Simultaneously, the same experimental design was replicated, this time exposing fish to 116 $\mu g.L^{-1}$ Roundup® (R group).

Tested concentrations of both commercial formulations were based on previous studies performed by the authors (Guilherme et al. 2010, 2012a; 2012b) (Guilherme et al.,

submitted), where these concentrations demonstrated to be able of exert genotoxicity in fish.

Fish were not fed during the exposure period, being daily fed with fish roe along the post-exposure period. Fish were sacrificed by cervical transection at the post-opercular region and blood collected from the heart using heparinised capillary tubes. Two µL of blood were immediately diluted in 1 mL of ice-cold phosphate-buffered saline (PBS) to prepare a cell suspension, which was kept on ice until further procedure.

### 7.2.3 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), in order to increase the assay output. Briefly, 20 µL of cell suspension (previously prepared in PBS) were mixed with 70 µL of 1% low melting point agarose, in distilled water. Eight drops of 6 µL were placed onto the precoated slide as two rows of 4 (4 groups of 2 replicates), without coverslips, containing each gel approximately 1500 cells. The gels were left for ±5 min at 4 ºC in order 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 ºC, for 1 h. After lysis of agarose-embedded cells, slides were washed 3 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 ºC.

Three sets of slides were prepared: two sets were incubated with endonucleases FPG or EndoIII, that convert oxidised purines and pyrimidines into DNA single strand breaks, respectively (Azqueta et al. 2009), and a third set was incubated only with buffer. Hence, 30 µL of each enzyme (diluted in buffer) were applied in each gel, along with a coverslip, prior to incubation at 37 ºC 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 $\mu g.L^{-1}$).

Fifty nucleoids were observed per gel, using a Leica DMLS fluorescence microscope (400x 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 this formula:

GDI = [(% nucleoids class 0)×0] + [(% nucleoids class 1)×1] + [(% nucleoids class 2)×2] + [(% nucleoids class 3)×3] + [(% nucleoids class 4)×4]

GDI values were expressed as arbitrary units in a scale of 0 to 400 per 100 scored nucleoids (as average value for the 2 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 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. In order to better estimate the overall magnitude of oxidative DNA damage, the sum of GDI with additional DNA breaks corresponding to both net FPG- ($NSS_{FPG}$) and EndoIII-sensitive sites ($NSS_{EndoIII}$) was also calculated ($GDI_{FPG+EndoIII}$). Moreover, the frequency of nucleoids observed in each comet class considering $GDI_{FPG}$ and $GDI_{EndoIII}$ was also expressed, as recommended by Azqueta et al. (2009).

#### 7.2.4 Statistical analysis

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

## 7.3 Results

### 7.3.1 Garlon® experiment

#### 7.3.1.1. Non-specific DNA damage

The analysis of the exposure period revealed a significant increase of the damage exerted by Garlon, when compared to the control group (Fig. 1A). The post-exposure period (Fig. 1A) showed that fish previously treated with Garlon still express significantly

higher damage values than the respective controls, for all sampling times. Moreover, the G group relative to 14 days showed a significant decrease in relation to the other G groups (1 and 7 days post-exposure). Significant effects of the factors agent and time were observed (Table 1) (2-way ANOVA results).

**Table 1.** Results of two-way ANOVA testing the effect of agent and time, as well as the interaction between them (agent x time) on the levels of DNA damage in blood cells of *A. anguilla* in a post-exposure period (1, 7 and 14 days), following a 3 days exposure to 270.5 µg.L$^{-1}$ Garlon®. Both F and p values are given for each variable. Non significant differences are signalized as "ns".

| Parameter | Factors | | | | Interaction | |
|---|---|---|---|---|---|---|
| | Agent | | Time | | Agent x Time | |
| | *F* | *p* | *F* | *p* | *F* | *p* |
| GDI | 92.33 | <0.05 | 20.94 | <0.05 | 2.15 | ns |
| $GDI_{FPG}$ | 33.14 | <0.05 | 16.20 | <0.05 | 7.67 | <0.05 |
| $NSS_{FPG}$ | 1.53 | ns | 1.16 | ns | 0.31 | ns |
| $GDI_{EndoIII}$ | 34.21 | <0.05 | 10.49 | <0.05 | 3.51 | <0.05 |
| $NSS_{EndoIII}$ | 0.12 | ns | 0.68 | ns | 1.01 | ns |
| $GDI_{FPG+EndoIII}$ | 27.45 | <0.05 | 2.09 | ns | 2.88 | ns |

7.3.1.2. Specific 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 and EndoIII (Figs. 1B-1E).

*FPG associated damage*

Similarly to what was observed for GDI in the exposure period, the $GDI_{FPG}$ parameter revealed a significant increase of damage for G group, in comparison with control (Fig. 1B). The post-exposure period (Fig. 1B) displayed this kind of difference only between groups relative to 1 and 7 days. The comparison between groups previously exposed to Garlon revealed a significant damage decrease after 14 days when compared to 1 and 7 days post-exposure. The $NSS_{FPG}$ parameter did not display any significant variation, either considering the exposure or the post-exposure periods (Fig. 1C).

Table 1 revealed, for $GDI_{FPG}$, a significant effect of agent and time, as well as a significant interaction between them (agent x time). $NSS_{FPG}$ showed to be unaffected by these factors.

The DNA damage classes were individually analyzed concerning the $GDI_{FPG}$ parameter (Table 2). The exposure period showed significant differences concerning all classes (excluding class 0), in relation to control. Moreover, the control group displayed class 2 as the most frequent, while G group presented a higher number of nucleoids corresponding to class 3. Looking the post-exposure period, it was evident a significant increase of class 4 for group G, in relation to control, concerning the day 1. The day 7 displayed a significant decrease in class 2 while an increase was detected for class 4, when G group was compared to control. In the fourteenth day, G group presented a general decrease of the damage in relation to 1 and 7 days, reflected in the increase of class 1 frequency, in parallel with the decreases of classes 3 and 4. At this point in time, class 2 was found as the predominant in both control and G groups.

*EndoIII associated damage*

In accordance with GDI and $GDI_{FPG}$, the $GDI_{EndoIII}$ parameter (Fig. 1D) pointed G group as presenting significantly higher damage than control, when the exposure period was considered. Concerning the post-exposure period (Fig. 1D), G groups corresponding to 1 and 14 days kept damage levels significantly higher than the respective controls. In line with what was described to the $NSS_{FPG}$, $NSS_{EndoIII}$ parameter (Fig. 1E) was unable to distinguish test groups, in any time.

Factors as agent and time, as well their interaction (agent x time) showed to have a significant effect on $GDI_{FPG}$ (Table 1). On the other hand, $NSS_{FPG}$ didn't display any significance.

Looking to the individual analysis of DNA damage classes during the exposure period, and concerning the $GDI_{EndoIII}$ parameter (Table 3), it was possible to notice that G group expressed a significant decrease in class 1, while classes 2 and 3 showed significant decreases, when compared to control. The control group showed class 2 as predominant and G group presented class 3 as the most frequent. The post-exposure period did not present significant alterations. Moreover, both control and G groups pointed class 2 as the predominant, with the exception of G group, in day 7.

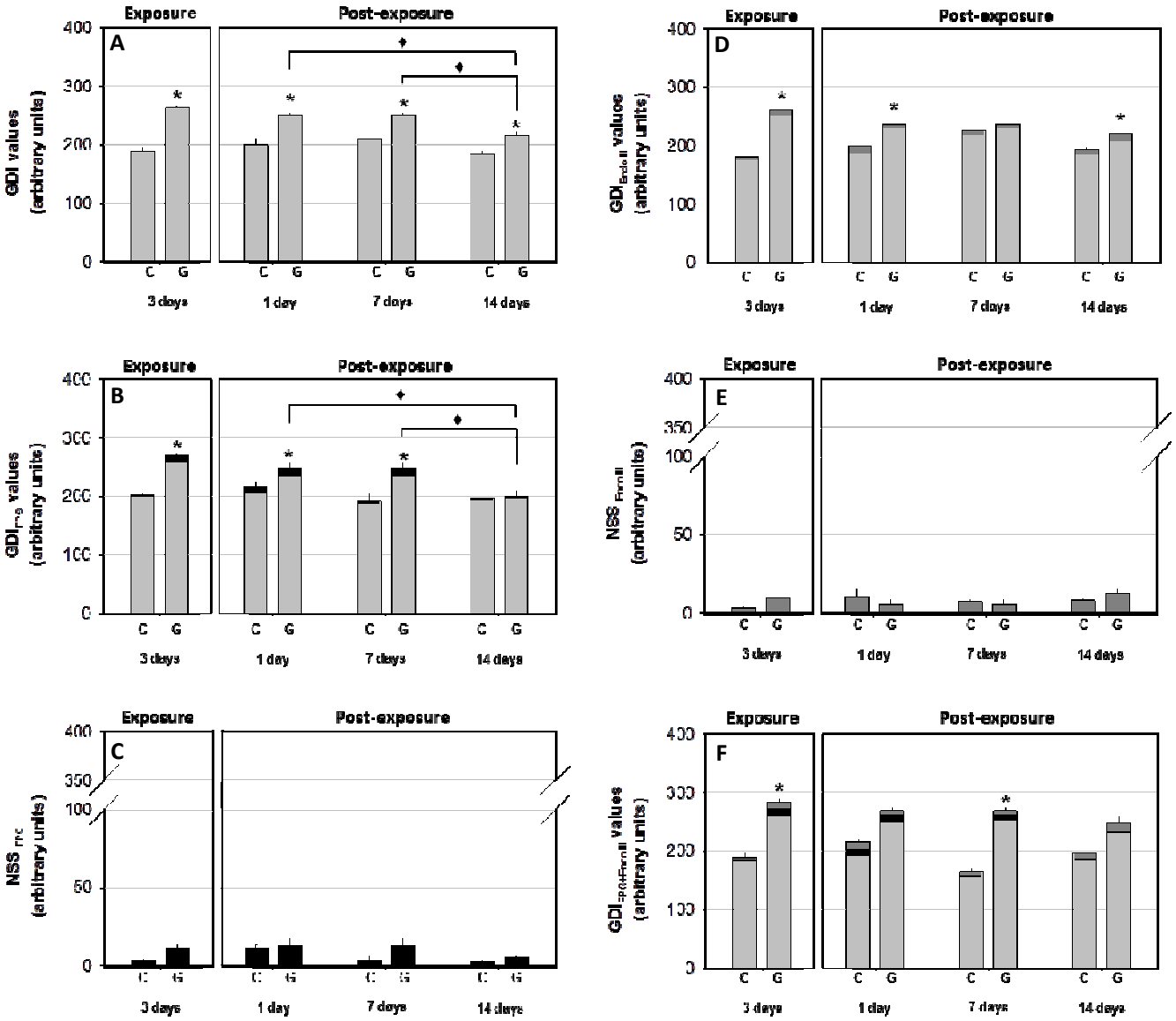


**Fig 1.** Mean values of DNA damage, measured by comet assay in blood cells of *A. anguilla* exposed to 270.5 µg.L$^{-1}$ Garlon® during 3 days (Exposure) and submitted to a period in clean water, during 1, 7 and 14 days (Post-exposure); (A) genetic damage indicator (GDI) measured by the standard (alkaline) comet assay; (B) overall damage ($GDI_{FPG}$) and partial scores, namely genetic damage indicator (GDI; grey) and additional DNA breaks corresponding to net FPG-sensitive sites ($NSS_{FPG}$; black); (C) $NSS_{FPG}$ alone; (D) overall damage ($GDI_{EndoIII}$) and partial scores, namely genetic damage indicator (GDI; light grey) and additional DNA breaks corresponding to net EndoIII-sensitive sites ($NSS_{EndoIII}$; dark grey); (E) $NSS_{EndoIII}$ alone; (F) sum of genetic damage indicator (GDI; light grey) with additional DNA breaks corresponding to net FPG-sensitive sites ($NSS_{FPG}$; black) and net EndoIII-sensitive sites ($NSS_{EndoIII}$; dark grey). Bars represent the standard error. Statistically significant differences ($p<0.05$) are: (*) in relation to control (C), within the same exposure time; (♦) between exposure times, within the same treatment.

*Overall oxidative damage*

Concerning the exposure period, the $GDI_{FPG+EndoIII}$ parameter (Fig. 1F) revealed significantly higher values for group G comparing to control. Looking to the post-exposure period (Fig. 1F), this difference was only evident for day 7.

Table 1 depicted the influence of factor agent

**Table 2.** 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 270.5 µg.$L^{-1}$ Garlon® during 3 days (Exposure) and upon cessation of exposure (1, 7 and 14 days post-exposure). Statistically significant differences ($p<0.05$) are: (*) in relation to control (C), (♦) in relation to 1 day post-exposure and (▲) in relation to 7 days post-exposure.

| | | | $GDI_{FPG}$ - DNA Damage Classes - Garlon® experiment | | | | |
|---|---|---|---|---|---|---|---|
| | | | 0 | 1 | 2 | 3 | 4 |
| Exposure | 3 days | C | 0.00±0.00 | 12.25±1.84 | 67.50±1.22 | 20.25±0.61 | 0.00±0.00 |
| | | G | 0.00±0.00 | 0.42±0.22* | 36.08±2.62* | 57.67±1.59* | 5.83±1.26* |
| Post-Exposure | 1 day | C | 0.00±0.00 | 8.00±3.27 | 66.92±1.29 | 24.83±4.76 | 0.25±0.20 |
| | | G | 0.00±0.00 | 0.58±0.08 | 55.00±4.80 | 43.67±5.22 | 2.00±0.25* |
| | 7 days | C | 0.00±0.00 | 14.75±0.61 | 55.83±0.95 | 27.42±1.56 | 2.00±0.00 |
| | | G | 0.00±0.00 | 1.00±0.52* | 48.17±3.25 | 45.17±2.57 | 5.67±0.36*♦ |
| | 14 days | C | 0.00±0.00 | 12.42±0.88 | 74.75±1.02 | 12.83±0.14 | 0.00±0.00 |
| | | G | 0.00±0.00 | 18.14±2.13♦▲ | 62.08±3.18 | 19.44±4.95♦▲ | 0.33±0.17♦▲ |

**Table 3.** 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 270.5 µg.L$^{-1}$ Garlon® during 3 days (Exposure) and upon cessation of exposure (1, 7 and 14 days post-exposure). Statistically significant differences ($p<0.05$) are: (*) in relation to control (C) and (▲) in relation to 7 days post-exposure.

| | | | $GDI_{EndoIII}$ - DNA Damage Classes - Garlon® experiment | | | | |
|---|---|---|---|---|---|---|---|
| | | | 0 | 1 | 2 | 3 | 4 |
| Exposure | 3 days | C | 0.00±0.00 | 24.25±1.02 | 66.75±0.61 | 9.00±0.41 | 0.00±0.00 |
| | | G | 0.00±0.00 | 0.92±0.92* | 40.58±3.25* | 53.42±3.60* | 5.08±1.54 |
| Post-Exposure | 1 day | C | 0.00±0.00 | 19.75±0.20 | 61.25±1.84 | 19.00±2.04 | 0.00±0.00 |
| | | G | 0.17±0.17 | 6.17±3.55 | 52.08±2.24 | 40.08±4.26 | 1.50±0.66 |
| | 7 days | C | 0.00±0.00 | 5.00±0.82 | 53.25±7.55 | 40.42±7.28 | 1.33±1.09 |
| | | G | 0.00±0.00 | 2.36±1.50 | 45.03±4.47 | 50.72±5.13 | 1.89±0.75 |
| | 14 days | C | 0.00±0.00 | 14.25±5.10 | 72.67±4.63 | 13.08±0.48 | 0.00±0.00 |
| | | G | 0.00±0.00 | 10.31±0.39 | 61.14±3.98 | 27.31±3.52▲ | 1.25±0.88 |

### 7.3.2 **Roundup® experiment**

#### 7.3.2.1. Non-specific DNA damage

Analysing the exposure period, it was possible to notice that the DNA damage exerted by Roundup®, reflected by the GDI parameter (Fig. 2A), have significantly increased when compared to the control group. Considering the post-exposure period (Fig. 2A), only the R groups corresponding to days 1 and 7 post-exposure showed significant differences in relation to their respective control. Moreover, it was noticed that the group which remained 14 days in clean water revealed a significant damage decrease when compared to the other two R groups (1 and 7 days post-exposure).

Table 4 (2-way ANOVA results) revealed a significant effect of the factors agent and time on GDI levels, as well as a significant interaction agent x time.

**Table 4.** Results of two-way ANOVA testing the effect of agent and time, as well as the interaction between them (agent x time) on the levels of DNA damage in blood cells of *A. anguilla* in a post-exposure period (1, 7 and 14 days), following a 3 days exposure to 116 µg.L$^{-1}$ Roundup®. Both F and p values are given for each variable. Non significant differences are signalized as "ns".

| Parameter | Factors | | | | Interaction | |
|---|---|---|---|---|---|---|
| | Agent | | Time | | Agent x Time | |
| | *F* | *p* | *F* | *p* | *F* | *p* |
| GDI | 152.62 | <0.05 | 83.11 | <0.05 | 31.06 | <0.05 |
| $GDI_{FPG}$ | 55.02 | <0.05 | 21.44 | <0.05 | 3.03 | ns |
| $NSS_{FPG}$ | 3.94 | ns | 5.48 | <0.05 | 8.47 | <0.05 |
| $GDI_{EndoIII}$ | 30.97 | <0.05 | 4.21 | <0.05 | 7.81 | <0.05 |
| $NSS_{EndoIII}$ | 2.47 | ns | 16.25 | <0.05 | 17.48 | <0.05 |
| $GDI_{FPG+EndoIII}$ | 33.71 | <0.05 | 3.05 | ns | 1.21 | ns |

7.3.2.2. Specific oxidative DNA damage (Figs. 2B-E).

*FPG associated damage*

The $GDI_{FPG}$ parameter (Fig. 2B) exhibited an increase of the DNA damage induced by Roundup®, after the exposure period (3 days). When fish previously exposed to Roundup® were transferred to clean water during 1, 7 and 14 days (Fig. 2B), significant increases were also perceptible for all R groups comparing to the respective control groups. However, the R group relative to 1 day post-exposure displayed significantly higher values when compared with R groups corresponding to 7 and 14 days post-exposure. On the other hand, the $NSS_{FPG}$ parameter (Fig. 2C) was only able to demonstrate significant differences in the post-exposure period. Thus, a significant increase was found comparing R and control groups at day 14. Moreover, at this time, the R group showed a significant $NSS_{FPG}$ increase in comparison to the corresponding groups at 1 and 7 days post-exposure.

Table 4 revealed, for $GDI_{FPG}$, a significant effect of agent and time. $NSS_{FPG}$ showed to be significantly affected by time and displayed a significant interaction agent x time.

The damage classes were also analysed individually, considering the $GDI_{FPG}$ parameter (Table 5). During the exposure period, class 1 displayed a decrease, while classes 2, 3 and 4 showed higher values, in relation to control. Moreover, it was observed that R group showed the prevalence of class 3, while control group displayed class 2 with

the highest frequency. After the first day of post-exposure, the R group, displayed a significant decrease in class 2, while classes 3 and 4 assumed values higher than control. The comparison between 1- and 7-day showed a significant increase concerning class 2 and displayed lower values for class 3. The fourteenth day presented a significant decrease between control and R groups, concerning the class 2. Moreover, all the classes (once again with the exception of class 0) concerning R group showed to be significantly different from the ones corresponding to the first day. Increases were perceptible considering classes 1 and 2, while classes 3 and 4 presented lower values. Considering the post-exposure period, class 2 was the most frequent with the exception of R group, in day 1.

*EndoIII associated damage*

Considering the exposure period to Roundup®, the $GDI_{EndoIII}$ parameter (Fig. 2D) displayed a significant increase for R group, in comparison with control. In what concerns to the post-exposure period (Fig. 2D), this significant increase was only detected for 1 and 14 days. Moreover, the R group corresponding to day 1 post-exposure assumed significantly higher values than the R groups at 7 and 14 days. The $NSS_{EndoIII}$ parameter (Fig. 2E) displayed a significant damage increase for the R group, when compared with control, considering the exposure period. The post-exposure period (Fig. 2E) revealed exactly the same pattern already described for $NSS_{FPG}$ parameter.

Table 4 revealed, for $GDI_{EndoIII}$, a significant effect of agent and time, as well as a significant interaction agent x time. $NSS_{EndoIII}$ showed to be significantly affected by time and displayed a significant interaction agent x time.

The analysis of individual damage classes in the exposure period, considering the $GDI_{EndoIII}$ parameter (Table 6), revealed the same pattern observed for $GDI_{FPG}$. After the first day of post-exposure, differences between control and R groups revealed a decrease in class 1, while class 3 presented higher values. The fourteenth day R group displayed a significant increase in class 1, as well as a significant decrease concerning class 3, when compared to its homologous in day 1. As observed for $GDI_{EndoIII}$, class 2 was presented as the prevalent for both groups, with the only exception for R group, in day 1.

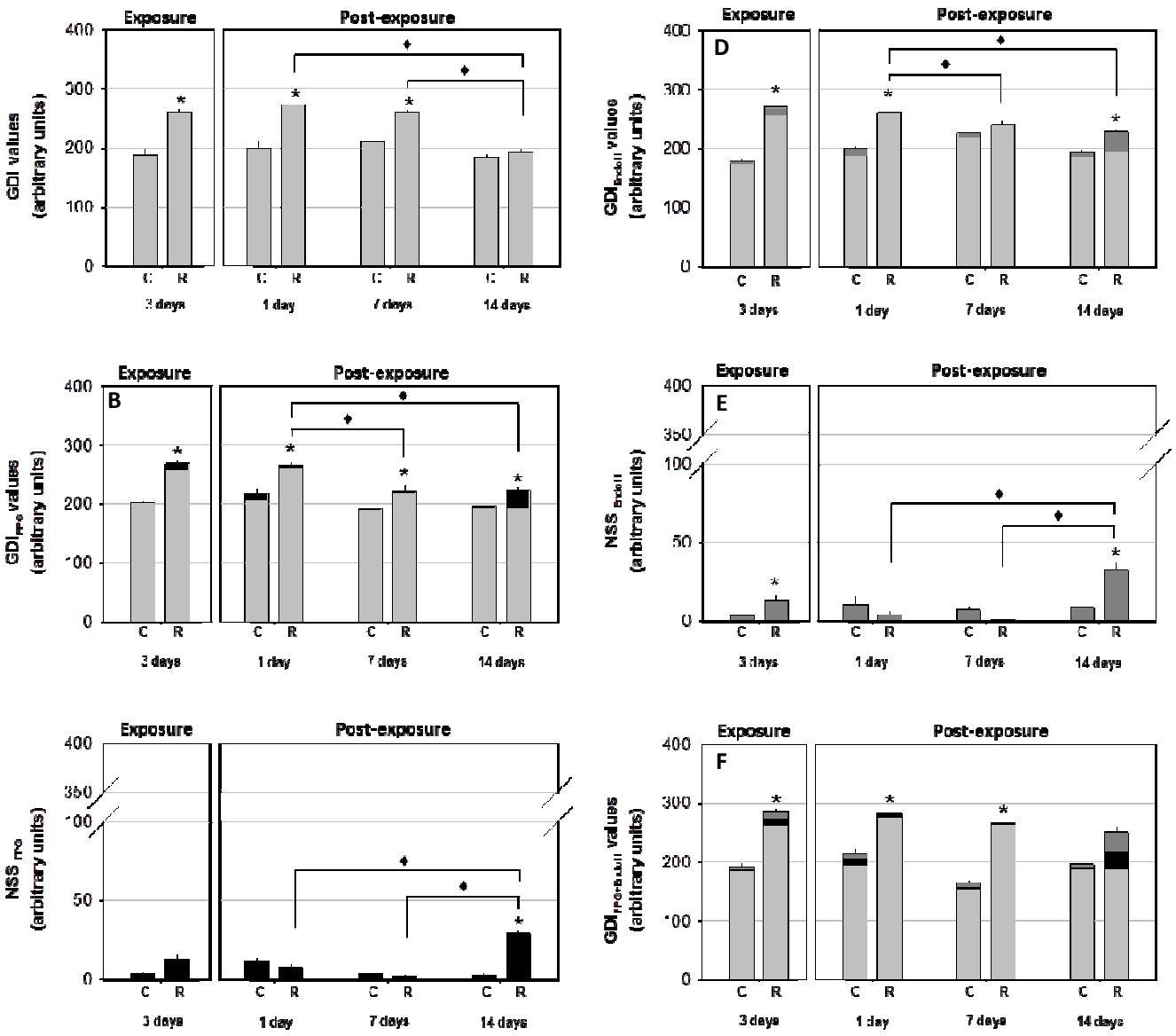


**Fig 2.** Mean values of DNA damage, measured by comet assay in blood cells of *A. anguilla* exposed to 116 µg.L$^{-1}$ Roundup® during 3 days (Exposure) and submitted to a period in clean water, during 1, 7 and 14 days (Post-exposure); (A) genetic damage indicator (GDI) measured by the standard (alkaline) comet assay; (B) overall damage ($GDI_{FPG}$) and partial scores, namely genetic damage indicator (GDI; grey) and additional DNA breaks corresponding to net FPG-sensitive sites ($NSS_{FPG}$; black); (C) $NSS_{FPG}$ alone; (D) overall damage ($GDI_{EndoIII}$) and partial scores, namely genetic damage indicator (GDI; light grey) and additional DNA breaks corresponding to net EndoIII-sensitive sites ($NSS_{EndoIII}$; dark grey); (E) $NSS_{EndoIII}$ alone; (F) sum of genetic damage indicator (GDI; light grey) with additional DNA breaks corresponding to net FPG-sensitive sites ($NSS_{FPG}$; black) and net EndoIII-sensitive sites ($NSS_{EndoIII}$; dark grey). Bars represent the standard error. Statistically significant differences ($p<0.05$) are: (*) in relation to control (C), within the same exposure time; (♦) between exposure times, within the same treatment.

*Overall oxidative damage*

The parameter $GDI_{FPG+EndoII}$ (Fig. 2F) signalized, for the exposure period, a significant damage increase for the group exposed to Roundup® (R group), in comparison to control. Concerning the post-exposure period (Fig. 2F), significant increases in R groups compared to the respective control groups were found after 1 and 7 days. In addition, $GDI_{FPG+EndoIII}$ showed a significant effect of the factor agent (Table 4).

**Table 5.** 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 116 µg.L$^{-1}$ Roundup® during 3 days (Exposure) and upon cessation of exposure (1, 7 and 14 days post-exposure). Statistically significant differences ($p<0.05$) are: (*) in relation to control (C), (♦) in relation to 1 day post-exposure and (▲) in relation to 7 days post-exposure.

| | | | $GDI_{FPG}$ - DNA Damage Classes - Roundup® experiment | | | | |
|---|---|---|---|---|---|---|---|
| | | | 0 | 1 | 2 | 3 | 4 |
| Exposure | 3 days | C | 0.00±0.00 | 12.25±1.84 | 67.50±1.22 | 20.25±0.61 | 0.00±0.00 |
| | | R | 0.00±0.00 | 0.42±0.30* | 36.58±3.24* | 56.92±0.79* | 6.08±2.21* |
| Post-Exposure | 1 day | C | 0.00±0.00 | 8.00±3.27 | 66.92±1.29 | 24.83±4.76 | 0.25±0.20 |
| | | R | 0.00±0.00 | 0.58±0.36 | 36.21±3.36* | 59.58±4.13* | 3.63±0.83* |
| | 7 days | C | 0.00±0.00 | 14.75±0.61 | 55.83±0.95 | 27.42±1.56 | 2.00±0.00 |
| | | R | 0.00±0.00 | 8.08±2.29 | 63.33±4.67♦ | 26.92±6.44♦ | 1.67±0.60 |
| | 14 days | C | 0.00±0.00 | 12.42±0.88 | 74.75±1.02 | 12.83±0.14 | 0.00±0.00 |
| | | R | 0.00±0.00 | 10.22±1.54♦ | 57.06±0.47*♦▲ | 32.33±1.96♦ | 0.39±0.20♦ |

**Table 6.** 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 116 µg.L$^{-1}$ Roundup® during 3 days (Exposure) and upon cessation of exposure (1, 7 and 14 days post-exposure). Statistically significant differences ($p<0.05$) are: (*) in relation to control (C) and (♦) in relation to 1 day post-exposure.

| | | | $GDI_{EndoIII}$ - DNA Damage Classes - Roundup® experiment | | | | |
|---|---|---|---|---|---|---|---|
| | | | 0 | 1 | 2 | 3 | 4 |
| Exposure | 3 days | C | 0.00±0.00 | 24.25±1.02 | 66.75±0.61 | 9.00±0.41 | 0.00±0.00 |
| | | R | 0.00±0.00 | 0.17±0.17* | 33.92±0.60* | 59.67±0.51* | 6.25±0.25* |
| Post-Exposure | 1 day | C | 0.00±0.00 | 19.75±0.20 | 61.25±1.84 | 19.00±2.04 | 0.00±0.00 |
| | | R | 0.00±0.00 | 0.75±0.38* | 40.42±4.06 | 54.75±4.94* | 4.08±1.31 |
| | 7 days | C | 0.00±0.00 | 5.00±0.82 | 53.25±7.55 | 40.42±7.28 | 1.33±1.09 |
| | | R | 0.00±0.00 | 4.83±0.96 | 53.92±3.84 | 37.42±3.47 | 3.83±1.31 |
| | 14 days | C | 0.00±0.00 | 14.25±5.10 | 72.67±4.63 | 13.08±0.48 | 0.00±0.00 |
| | | R | 0.00±0.00 | 7.83±1.88♦ | 57.33±3.18 | 33.92±1.96♦ | 0.92±0.79 |

## 7.4 Discussion

The herbicides tested in the present study, though not extensively studied in terms of their genotoxic potential, have already been considered as DNA damaging agents to fish, which is an indispensable condition for pursuing the main objective of this work, *viz.* the evaluation of DNA damage progression along a post-exposure period. Hence, in a recent study of our research group, fish exposed to an environmentally relevant concentration of Garlon® (270.5 µg.L$^{-1}$) showed increased DNA damage assessed by the comet assay (Guilherme et al. submitted). However, when the additional breaks corresponding to net enzyme-sensitive sites were considered, no significant levels of oxidative damage were detected (Guilherme et al. submitted). Concerning Roundup®, its genotoxicity to fish has been demonstrated by different authors, based on different genotoxic endpoints (Çavas and Könen 2007; Cavalcante et al. 2008; Guilherme et al. 2012b). The participation of oxidative stress on the damage induced by Roundup® was

evidenced by the detection of oxidatively altered DNA bases (by means of the enzyme-modified comet assay) both in liver and blood tissues (Guilherme et al. 2012a; 2012b).

In line with the previous findings, the genotoxic potential of both herbicides was confirmed by the current data concerning the exposure period (3 days). In addition, the apparent lower involvement of oxidative DNA damage on the action of Garlon® and the association of that damaging mechanism to the action of Roundup® ($NSS_{EndoIII}$ parameter pointed out the pyrimidines oxidation) were also confirmed.

The processes that determine DNA damage, and its extent, can be divided according to its intervention towards a genotoxicity promotion and, in opposition, according to its anti-genotoxic role. DNA integrity may be affected as a result of a direct attack of the genotoxicant (e.g. formation of DNA adducts) and/or its metabolites (Bonfanti et al. 1992), as well as, indirectly, through the over-generation of ROS that may disrupt DNA or affect the function of DNA repair enzymes (Shimura-Miura et al. 1999). In addition, a depletion of the overall antioxidant defences caused by the contaminant can create a pro-oxidant condition, which in turn also promotes DNA damage (Ahmad et al. 2006). On the other side, it can be considered the increment of DNA repair mechanisms and antioxidant actions as well as cell turnover in the target tissue. Keeping in mind the latter process, it should be considered that genetic damage manifestations (e.g. micronuclei, DNA strand breaks) and their detection can be affected by cellular renovation (Saleha Banu et al. 2001), which in turn can also be modulated by the genotoxic agent itself (Pacheco and Santos 2002). Therefore, the occurrence/absence of a significant DNA damage in a given moment depends on the balance between the two types of processes above mentioned. An eventual recovery of damage in a post-exposure period relies on the tendency of the anti-genotoxic processes to gain preponderance in relation to the genotoxic pressures because of the cessation of exposure. Nevertheless, it cannot be overlooked the hypothesis that post-exposure period can be more critical, as demonstrated in fish exposed to a recognised genotoxicant (benzo(a)pyrene) (Rose et al. 2001; Nigro et al. 2002).

The following discussion will be focused on the evolution of damage after removal of the contamination source, bringing to the fore the main processes likely to be involved. This approach is innovative, since it has never been conducted in fish in the context of herbicides genotoxicity.

### 7.4.1 Progression of DNA damage induced by Garlon® in post-exposure period

In what concerns to the post-exposure period, it was possible to observe slightly different patterns depending on the parameter considered. Thus, the GDI parameter, although showing a tendency to damage decrease along this period, did not reflect an effective recovery from the damage previously induced, since the control levels were not restored. Reinforcing the weakly supported indication provided by GDI towards recovery, the $GDI_{FPG}$ parameter revealed an actual recovery 14 days after the cessation of exposure, which was substantiated by the damage decreases in relation to days 1 and 7 (also corroborated by the significant effect of the factor time). Differently, $GDI_{EndoIII}$ data cannot be interpreted as reflecting a recover. Despite the absence of a significant damage increase at day 7 (G *vs*. C groups), it was not possible to perceive neither a decrease tendency along the whole period nor a significant decrease in relation to R group at day 1. On the other hand, the analysis of individual classes of damage along the post-exposure period, either considering $GDI_{FPG}$ or $GDI_{EndoIII}$, displayed a general tendency of frequency increase for nucleoids of class 2, concomitantly to a decrease in class 3, which supports the idea of a damage extent reduction.

The interaction agent x time detected for $GDI_{FPG}$ and $GDI_{EndoIII}$ (two-way ANOVA) didn't prevent the confirmation of significant effects for these factors separately.

The analysis of the overall oxidative damage ($GDI_{FPG+EndoIII}$) showed a significant damage only at day 7 post-exposure. Considering that this parameter derives from the sum of GDI, $NSS_{FPG}$ and $NSS_{EndoIII}$ scores, it is important to underline that it reflected a response pattern different from each partial score that is on the basis of its calculation. Thus, contrarily to what was previously stated by the authors (Guilherme et al. 2012b), the analysis of this parameter revealed some limitations on the genotoxicity evaluation.

The submission of fish to a post-exposure period can be regarded as a simulation of its shifting from a contaminated to an uncontaminated area. This alteration ensures that the exposure to waterborne contaminants was abolished. However, the xenobiotic(s) previously absorbed can still persist in the organism's tissues, promoting the extension of damage beyond the exposure period. Bearing this in mind, it is important to understand the toxicokinetics and toxicodinamics, mainly, of the active ingredient of Garlon® (triclopyr). Usually, the half-life time of a pesticide is defined for its active ingredient. Thus, it is known that triclopyr needs less than a day to be reduced to 50% of its concentration in fish's body (Thompson et al. 1991). Considering this, the maintenance of a DNA damaging effect after the cessation of exposure, in the time scale currently assessed, cannot be associated to the persistence of triclopyr in fish's body and the subsequent

direct attack to DNA. Hence, the impossibility of eel's blood cells to fully recover from the damage induced by Garlon® reflected, mainly, the incapacity of DNA repair system to restore DNA integrity in a period of 14 days.

A negligible involvement of oxidative damage seems to be depicted in the absence of significant increases of $NSS_{FPG}$ and $NSS_{EndoIII}$ values. However, this assumption should be taken carefully, since, as mentioned above, a recovery of damage value up to the control level was exhibited by $GDI_{FPG}$ on day 14. Hence, the most plausible interpretation points to a minor induction of purine bases oxidation as a result of Garlon® exposure, which was not enough to produce statistical significances, but that shouldn't be ignored. In line with this interpretation, it became evident an efficient intervention of DNA repair enzymes specifically directed to oxidized purine lesions.

### 7.4.2 Progression of DNA damage induced by Roundup® in post-exposure period

In what concerns to this period, GDI parameter revealed a decrease on the non-specific DNA damage induced by Roundup® after 14 days in clean water, while $GDI_{FPG}$ and $GDI_{EndoIII}$ failed to express a complete recovery of DNA stability.

Keeping in mind the recovery process evidenced by GDI, it should be first questioned if the suppression of a direct genotoxic pressure resulting from chemical elimination from the fish body could be on the basis of that damage decline. In this direction, it is known that glyphosate, the active ingredient of Roundup®, has a low potential to bioaccumulate (WHO 1994). Therefore, the reduction of glyphosate (or its metabolites) body burden, as an important condition to allow damage recovery, was probably achieved 14 days after the cessation of exposure.

A second question arises concerning the removal of damaged erythrocytes from circulation together with the production of new cells as a possible explanation for the absence of DNA damage (measured as GDI) on day 14. The life span of erythrocytes in fish is supposed to be around 100 days, but it can be reduced as a consequence of exposure to contaminants. In this direction, an increased splenic erythrophagia was already associated to an intense genetic damage in *A. anguilla* (Pacheco and Santos 2002). Nevertheless, the overall results did not support this hypothesis since an eventual influence of the erythrocytes population renovation would be also reflected in $GDI_{FPG}$ and $GDI_{EndoIII}$ scores, which didn't happen.

When the DNA damage measured as $GDI_{FPG}$ or $GDI_{EndoIII}$ is under consideration, a complete recovery process, as discussed above for GDI, is no more evident. It is particularly interesting to highlight that, analysing GDI *versus* $GDI_{FPG}$ or $GDI_{EndoIII}$, the

persistence of damage on day 14 reflected by the latter parameters was coincident with the late appearance of DNA breaks corresponding to net enzyme-sensitive sites (as depicted in $NSS_{FPG}$ and $NSS_{EndoIII}$ values). $NSS_{FPG}$ and $NSS_{EndoIII}$ results indicated the occurrence of oxidative damage on day 14 post-exposure, affecting both purine and pyrimidine bases. This is indicative of a progressive decay of cell antioxidant protection, favouring a pro-oxidant status, coupled with the incapacity to repair this particular type of DNA damage.

The lack of a significant effect of the factor agent for $NSS_{FPG}$ and $NSS_{EndoIII}$ data in two-way ANOVA reflected the complexity of processes involved, also depicted on the interaction agent x time detected.

It can be also inferred that, following Roundup® exposure, blood cells of *A. anguilla* were more successful in repairing damage detected by GDI (here called non-specific damage), than that associated to bases oxidation. However, some repair capacity was still evident when analysing the individual classes of damage, either considering $GDI_{FPG}$ or $GDI_{EndoIII}$, which displayed a general decrease tendency in the frequency of class 3 nucleoids along the post-exposure period (while class 2 frequency increase).

Therefore, it was demonstrated an indirect action of Roundup® on DNA, related to the formation of ROS, occurring in a late phase. This interaction can be due to the redox cycle of Roundup® constituents generating ROS and/or to the inhibition/exhaustion of the antioxidant defences, decreasing thus the ability to counteract a ROS challenge (even at basal levels). Supporting this idea, organophosphate pesticides affect DNA bases either directly or indirectly via protein alkylation (Mohan 1973). Additionally, and according to Saleha Banu et al. (2001), besides ROS dependent processes, organophosphate pesticides can also inhibit enzymes involved in DNA repair.

Taking into account the present overall results, only a partial recovery can be assumed concerning the genotoxic endpoints assessed. Even considering the hypothesis that fish can restore their DNA integrity measured by the comet assay in blood cells, a question remains whether a single exposure can lead to long-term genome-destabilizing effects and fixed in stable genotoxic lesions. The transient nature of chromosomal damage in the context of pesticides exposure has been assessed in human studies. Thus, patients suffering acute organophosphate intoxication evidenced an increase in the frequency of chromosome aberrations, which returned to normal levels 6 months after the acute exposure (van Bao et al. 1974). Another study revealed that the cessation of occupational exposure to phosphine was accompanied by a significant decline in the chromosome rearrangements frequency within 1 year time (Garry et al. 1992).

Subsequently, the genotoxic potential of Roundup® currently detected in *A. anguilla* should be regarded as a primary risk factor for long-term effects such as carcinogenesis. In line with this, it was reported a late appearance of long-term adverse effects (e.g. carcinogenicity and reproductive impairments) in fish exposed to pesticides (Kime 1995; Moore and Waring 2001; de Campos Ventura et al. 2008).

In an attempt to evaluate the suitability of the adopted genotoxic endpoints on the assessment of genotoxic hazard associated to the tested herbicides in fish environmentally exposed, it was demonstrated that comet assay (standard version and applying the extra step with DNA lesion-specific repair enzymes) applied to blood cells displayed promising aptitudes, namely reducing the possibility of false negatives results in a time scale of days/weeks in animals that are no longer exposed.

### 7.5 Conclusion

The present results confirmed the genotoxicity of the herbicide formulations Garlon® and Roundup® to fish, considering environmentally realistic concentrations.

Analysing the progression of DNA damage along the post-exposure period (up to 14 days), it was observed that fish exposed to Garlon®, though presenting some evidences towards a recovery tendency, didn't achieve a complete recovery. A recovery of damage was only exhibited by $GDI_{FPG}$ parameter (day 14), pointing to a minor induction of purine bases oxidation coupled with an efficient intervention of repair enzymes specifically directed to these DNA lesions.

In relation to Roundup®, a recovery was evident when considering non-specific DNA damage (GDI), on day 14 post-exposure, while $GDI_{FPG}$ and $GDI_{EndoIII}$ failed to express a complete restoration of DNA integrity. In addition, this glyphosate-based herbicide showed a potential to oxidatively damage DNA, depicted mainly as a late (day 14) appearance of DNA breaks corresponding to net enzyme-sensitive sites ($NSS_{FPG}$ and $NSS_{EndoIII}$). Moreover, blood cells of *A. anguilla* exposed to Roundup® were more successful in repairing damage detected by GDI (non-specific damage), than that associated to bases oxidation.

Overall, the present findings highlighted the genetic hazard to fish associated to the addressed agrochemicals, reinforcing the hypothesis of long-lasting damage.

**Ethical statement**

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.

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# Chapter VIII

**General Discussion**

# 8 General Discussion

This chapter aims to provide an integrated overview of the main outcomes of the chapters II to VII, contributing with new interpretative perspectives not presented in the inside discussion of each chapter. The current discussion was built taking into account the key objectives of the thesis and their throughout progression. The linkage to the general and specific objectives of the thesis and the contribution to the scientific knowledge in the area were also highlighted.

Clarifying the rational that directed the development of the present research, it should be noted that the work was focused on the herbicide formulations Roundup® and Garlon®, though more attention was devoted to the former. Hence, the chapters II to V were dedicated to Roundup®, the chapter VI to Garlon®, and the chapter VII to both. Besides the assessment of the effects of these formulations as a whole, in both cases the discrimination of the contribution of their ingredients to the final impact was also addressed (chapters IV for Roundup®; chapter VI for Garlon®). In the case of Roundup®, two additional perspectives were explored concerning the clarification of tissue-specific damage responses (chapters II and III) and the evaluation of the risk associated to the main environmental metabolite of the active principle (chapter V). The *A. anguilla* ability to recover from the DNA damage induced by short-term exposures to the herbicides upon the exposure cessation was approached for both formulations in the chapter VII, keeping in mind the understanding of the genotoxicity dynamics of these agrochemicals in fish. The improvement of the knowledge on the mechanisms underlying the measured genetic damage was a perspective transversal to chapters II to VII, namely as a contribution to elucidate the involvement of oxidative DNA damage. As another perspective present in all the research components (chapters II to VII), it appears the intention to contribute to the definition of biomonitoring tools towards an efficient assessment of the risk associated to the occurrence in the water systems of the selected agrochemicals. The perspectives now enunciated constituted the guidelines for the following discussion.

## 8.1 Genotoxic risk evaluation of the herbicide formulations

Regardless of the class to which herbicides belong, their presence in the aquatic environment is often related to pernicious effects at various levels (Clements et al. 1997; Brock et al. 2006; Crestani et al. 2007; da Fonseca et al. 2008), highlighting their toxicity. Despite the relevance of these effects in general, those that are related with the “molecule of life” play a primordial role in the toxicological field. DNA damage may promote short,

medium and long term effects, evidencing the importance of signalizing the chemicals that may compromise its viability/functionality.

The assessment of the genotoxic hazard posed to fish by the two different herbicide formulations - Roundup® and Garlon® - was one of the central objectives of this research, keeping in view their extensive use worldwide and the notorious scarcity of scientific information in this direction, which, at least by omission, has been allowing an unsupported idea of environmental safety.

The herbicides tested, although used with similar purposes in the agricultural context (Relyea 2005; Wojtaszek et al. 2005), present quite different characteristics, namely concerning their active ingredients. Regarding the chapters II to VI, it was clearly demonstrated that both commercial formulations were able of exert genotoxic effects in *Anguilla anguilla*. Short-term exposures (1 and 3 days) to environmentally realistic concentrations proved to be sufficient to point out the ability of both formulations in exert DNA damage (measured by the comet assay). However, the subjacent mechanisms concerning the ability of Roundup® and Garlon® to exert DNA damage revealed to be different, as will be exploited in a following point of this section. In addition, and considering the exposure to Roundup®, the induction of chromosomal damage (measured by the ENA assay) was also clear, despite only occurring after the third day of exposure.

Most of genotoxic studies in fish has been performed in erythrocytes (Ayllon and Garcia-Vazquez 2001; Grisolia and Starling 2001). However, the use of other cell types has been strongly suggested (Sharma et al. 2007) keeping in view the understanding of tissue-specific responses and thus achieving a better perspective about the overall condition of the organisms. Nonetheless, the tissue choice may not be consensual, due to specific features (advantages/drawbacks) of each cell types. Considering this, and in what concerns to Roundup®, the genotoxicity was demonstrated in three different tissues, i.e. blood, gills and liver (chapters II and III). Blood and gills were able to reflect the occurrence of damage along the whole experiment (1 and 3 days) and for both tested concentrations On the other hand, liver presented an adaptive behaviour to the genotoxic stimulus, being not able to signalize damage after 3-day exposure to the lower concentration of Roundup®. Thus, blood and gills appears as the most adequate choices to the evaluation of the genotoxic risk in environmental waters with moderate waterborne concentrations of this herbicide.

Excluding the few studies concerning the genotoxicity of Roundup® to fish (Grisolia 2002; Çavas and Könen 2007; Cavalcante et al. 2008), the genotoxic potential of both herbicides remained almost unexploited until date. Therefore, the present study strongly

contributed to a knowledge improvement on the hazard evaluation regarding their ability to exert genotoxic effects.

### 8.2 Formulations *versus* active ingredients – have they different genotoxic potentials?

The exposure to pesticides often involves complex mixtures of different types of chemicals, such as active ingredients and by-products present in technical formulations, as well as impurities, solvents, surfactants and other compounds frequently considered as inert ingredients. In a review of pesticides genotoxicity in human populations, carried out by Bolognesi (2003), it was clearly stated that, although inert ingredients have no pesticidal activity, they may be biologically active and sometimes the most toxic component of a pesticide formulation. In this direction, the present research had among its goals the elucidation of differential genotoxic potential of the commercial formulations and their constituents.

If on one hand, the different contribution of the each Roundup® ingredients was made possible by the availability of the formulation composition (chapter IV), on the other, in what concerns to Garlon®, it was only possible to assess the genotoxicity of its active ingredient due to the impossibility to come to know the detailed formulation (chapter VI). Contrary to what was expected based on the idea that glyphosate-based formulations are generally considered more toxic than pure glyphosate (Rank et al. 1993; Tsui and Chu 2003; Peixoto 2005), and looking to the non-specific DNA damage, the Roundup® formulation appeared to be, most of the time, less genotoxic than its active ingredient glyphosate or the surfactant alone. Furthermore, fish exposed to Roundup® presented levels of damage lower than expected, based on the sum of the effects of the separate components, pointing an antagonism between their constituents. However, when the oxidative potential was evaluated, and looking to the breaks associated to oxidized purines ($NSS_{FPG}$), a synergistic interaction was perceptible between glyphosate and the surfactant POEA, highlighting the Roundup® hazard (chapter IV). In addition to the genotoxic evaluation of the formulation and its components, the metabolite resulting from the degradation of the active ingredient also showed to be a concern. In this way, AMPA showed its ability in exert either DNA or chromosomal damage. Hence, and considering these findings, the inclusion of AMPA in studies concerning the risk assessment of glyphosate-based herbicides is strongly recommended.

In a different way, Garlon® showed a higher genotoxic potential when compared to its active ingredient triclopyr (chapter VI). Therefore, the possible interference of the unknown surfactant (omitted in the technical information provided by the manufacturer), as well as the adjuvant (querosene), despite generally considered as ‘‘dilutant’’ or ‘‘inert”, cannot be neglected.

Bearing this in mind, it seems important to consider some alternatives in order to minimize the adverse effects exerted by these herbicides, namely encouraging the development and increasing use of more ecological formulations including less toxic adjuvants. Moreover, the label detailed description considering all the mixture constituents appears as a priority, as well as a further revision concerning their individual hazard classification. In the specific case of Roundup®, some alternatives to surfactants are under development, in order to reduce toxicity or even avoid the use of a surfactant (for instance Tornado®). Nevertheless, it must be noted the need to thoroughly test the new products with sensitive methodologies as those now adopted, in order to fully evaluate their genotoxic risk.

In what concerns to Garlon®, the eventual alteration of the formulation so that the adjuvants and/or surfactants do not increase the toxicity of the active ingredient appears as a positive measure.

### 8.3 Contribution to the clarification of DNA damaging mechanisms

One of the main goals of the present thesis was to clarify the mechanisms of damage subjacent to the herbicides genotoxic action. Accordingly, two different methodologies were applied: the comet and ENA assays. The comet assay measures DNA stand breaks, a repairable type of damage, while the ENA assay points out chromosomal lesions, signalizing a type of damage difficult of being repaired. The chapters II and V covered both genotoxic endpoints, considering different agents (commercial formulation Roundup® and the breakdown product of glyphosate – AMPA). Thus, both approaches indicated the genotoxic potential of the mentioned agents. The ENA assay reflected a late appearance of damage (when compared to comet assay), while the comet assay demonstrated to be able to point out the damage occurrence in a short period after the exposure, denoting an early nature of the damaging events. The results also showed that only ENA assay was able to distinguish between tested concentrations (considering both tested agents). The two parameters showed a positive correlation in Roundup® exposed fish, while the AMPA experiment revealed contradictory

results. Therefore, a hypothetical relation between the occurrence of DNA and chromosomal damage couldn't be entirely demonstrated. Bearing this in mind, the importance of their jointly application was confirmed, since they provide complementary information, allowing a more effective genotoxic risk assessment.

Chapters II to VII contributed to clarify the mechanisms underlying the genetic damage derived from exposures to Roundup® and Garlon® and their related compounds. In chapters II and III, the oxidative stress was hypothesized as a potential mechanism of damage exerted by Roundup®. Results concerning the blood and liver tissues revealed that neither enzymatic nor non-enzymatic antioxidant defenses provided evidence of a pro-oxidant challenge. On the other hand, gills pointed a punctual increase in CAT, signalizing an overproduction of $H_2O_2$, which may indicate that the DNA oxidation may play a role on the Roundup® capacity to exert genotoxicity. Another approach to elucidate the involvement of DNA oxidation in the genotoxicity of Roundup® and Garlon® and their components/by-products was adopted concerning the standard comet assay methodology upgraded with an extra-step involving incubation with repair enzymes (FPG and EndoIII) (chapters III to VII). This approach intended to clarify the eventual direct attack to DNA due to the presence of ROS (chapter IV and VII) even if the antioxidant system showed to be unresponsive, as observed in the hepatic cells after exposure to Roundup® (chapter III). Considering this, the oxidation of DNA might be considered as an underlying mechanism to the Roundup® genotoxicity (even when the threshold limit to induce the antioxidant system was not reached). This mode of action of Roundup® was predictable since the pro-oxidant properties of the organophosphate pesticides were already known (Banerjee et al. 2001). In relation to Garlon® and its active ingredient triclopyr (chapter VI), no significant levels of DNA oxidative damage were detected.

Chapter VII intended to shed light in the transience and/or reversibility of DNA-damaging effects, as an attempt to clarify the mechanisms involved after cessation of the exposure. Considering the Roundup® experiment, the post-exposure period revealed a decrease concerning the non-specific DNA damage (expressed by GDI parameter), but the recovery process was not expressed by $GDI_{FPG}$ and $GDI_{EndoII}$ parameters, since the persistence of damage was highlighted by the late appearance of DNA breaks corresponding to net-enzyme sensitive sites. Considering this, a progressive decay of cell antioxidant protection as well as the incapacity to repair this particular type of damage was suggested. Chapter VII also aimed the clarification of mechanisms determining Garlon® effects in a post-exposure period. Despite a tendency to damage decrease along

this period depicted by the GDI parameter, it couldn't be regarded as an effective recovery. This slight indication was reinforced by the $GDI_{FPG}$ parameter, which revealed an effective recovery after 14 days of post-exposure. Thus, it became evident an efficient intervention of DNA repair enzymes specifically directed to oxidized purines. On the other hand, $GDI_{EndoIII}$ pointed out the damage maintenance along the post-exposure period. The most plausible interpretation pointed to a minor induction of purine bases oxidation as a result of Garlon® exposure, suggesting that the indication towards limited oxidative potential of this formulation presented in chapter VI requires further confirmation.

### 8.4 Implications for the definition of biomonitoring strategies

The biomonitoring strategies should be able to assess the condition of individuals in key moments, reflecting the extent and persistence of a given environmental risk. In this direction, the present research reinforced the usefulness of both genotoxic endpoints adopted as biomonitoring tools applied to fish in the detection of a genotoxic pressure resulting from the occurrence of herbicides in water systems. The standard comet assay proved to be a suitable tool applied to different fish tissues, being its sensitivity improved with the inclusion of DNA lesion-specific repair enzymes. The ENA assay also proved its ability to signal herbicide-induced damage, being its use recommended mainly in combination with comet assay.

The intermittence of herbicides input in the water systems may represent an additional challenge to biomonitoring programs using wild specimens, since fish can be sampled in a moment that are no more under the action of the agrochemicals. In this context, it becomes determinant the knowledge about the persistence of key biological responses after cessation of exposure, which also means the knowledge on the possibility of fish responses to reflect recent contamination episodes. As a contribute in this direction, the present work (chapter VII) revealed that DNA integrity loss in blood cells associated to the tested herbicide formulations and measured by the comet assay remained for at least 14 days after cessation of exposure. This finding provided an additional substantiation towards the inclusion of this genotoxic endpoint in biomarker batteries in the framework of Ecological Risk Assessment (ERA) of pesticides contaminated areas. Moreover, the comet assay with DNA lesion-specific repair enzymes showed to represent a value-added in this context since it was able to detect a late appearance of oxidative DNA damage in the post-exposure period.

In the context of biomonitorization, it was stated by van der Oost (2003) that fish are generally considered as the most feasible organisms for pollution monitoring in aquatic systems. Their great sensibility allows signalling environmental risk, which may be then extrapolated to other species. Due to its characteristics, the European eel (*Anguilla anguilla* L.) have been successfully used is several studies in the evaluation of genotoxicity (Pacheco et al. 1993; Pacheco and Santos 1996, 1997, 1998, 2002; Ahmad et al. 2006; Oliveira et al. 2008). The high fat content and benthic feeding habits in continental waters make the eel vulnerable to the bioaccumulation of pollutants. The use of *A. anguilla* as test-organism in the present study confirmed its potential to perform this kind of approaches. Its sensitivity to genotoxicants allowed to detect responses to low contamination levels (in a range found in the environment), as well as to distinguish between different contaminant concentrations. In addition, it was also perceptible a tissue-specificity, contributing to a more robust assessment concerning the toxicodinamics/toxicokinetics of the genotoxicant.

Throughout the present study, both yellow (II and III) and glass (chapters IV to VII) eel stages were adopted. The alteration of the life stage was carried out, mainly, due to fact that glass eel facilitates the implementation of the experimental designs as a result of its small size. Hence, it was not intended to carry out a comparison of the effects in the two life stages, though that question is of interest and could be exploited in the future.

### 8.5 Final Remarks

The present research brought new perspectives concerning the hazard posed by herbicides to aquatic organisms. Thus, the genotoxicity to fish of both Roundup® and Garlon® formulations, at environmental concentrations, was undoubtedly recognized. In an attempt to identify the contribution of their constituents to the overall effect, it was demonstrated that, in the case of Roundup®, glyphosate and the surfactant POEA have comparable genotoxic potentials, though the sum of their individual effects was never observed. This observation emphasized the need to define regulatory thresholds for all the formulation components, recommending, in particular, the revision of the hazard classification of POEA (classified as “inert” until date). In what concerns to Garlon®, though triclopyr displayed a genotoxic potential individually, the formulation showed to be more genotoxic than the active ingredient.

As an innovative perspective in the context of the assessment of herbicides impact, it was demonstrated that the products of environmental degradation of the active

ingredients can constitute a silent problem. This was highlighted by the observation that AMPA - the major breakdown product of glyphosate - has a genotoxic potential similar to its precursor.

The comet and ENA assays were performed in order to differentiate the types of damage (DNA and chromosomal damage, respectively) exerted by the mentioned herbicides and their related products. The comet assay showed to reflect early damaging events in comparison with ENA assay. The latter endpoint showed a better capacity to discriminate tested concentrations. Hence, their jointly application is strongly recommended as they provide complementary information.

The present findings also allowed understanding tissue-specificities concerning the DNA damage, with gills and blood proving to be more adequate to genotoxic evaluation, when compared to liver, due to their higher responsiveness.

The comet assay upgraded with an extra-step involving incubation with repair enzymes (FPG and EndoIII) pointed out the oxidative DNA damage exerted namely by the formulation Roundup®. The standard comet procedure, detecting only non-specific DNA damage, displayed some limitations when compared to the previous methodology that reduced the possibility of false negative results. In relation to Garlon® and triclopyr, DNA oxidation was not perceived as a dominant mechanism of damage.

The assessment of genotoxic endpoints in *A. anguilla* after removal of the contamination source revealed, for both formulations, a fish incapacity to completely recover in a period of 14 days. It was also recognized that blood cells of fish exposed to Roundup® were more successful in repairing damage with a non-specific cause, than that associated to bases oxidation. These findings emphasized the genetic hazard associated to the addressed agrochemicals, reinforcing the hypothesis of long-lasting damage.

Overall, the results obtained provided useful recommendations for policy-making, contributing to (re)formulate regulatory procedures for protecting the health of aquatic environment. In this direction, the data gathered in this work point to the importance of performing a genotoxic evaluation in order to actually determine the hazard posed by herbicides and their by-products. The magnitude of risk detected for both formulations strongly advise the adoption of restrictive measures in relation to their application in the proximity of watercourses. As mitigation measures, the development of formulations incorporating adjuvants selected on the basis of their lower toxicity emerged as a recommended path.

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