Universidade de Aveiro Departamento de Biologia
2013

Ana Margarida Lourenço Silva Marques

Dano e reparação de ADN em *Anguilla anguilla* L. exposta a pesticidas

DNA damage and repair in *Anguilla anguilla* L. exposed to pesticides

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, realizada sob a orientação científica do Doutor Mário Guilherme Garcês Pacheco, Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro

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palavras-chave

Pesticidas, contaminação aquática, *Anguilla anguilla*, Decis®, Roundup®, genotoxicidade, reparação de ADN.

resumo

A ocorrência de pesticidas no meio aquático tornou-se uma questão ambiental de elevada preocupação, considerando que esta vasta classe de compostos pode induzir um leque variado de efeitos nefastos em organismos aquáticos, nomeadamente em peixes. A sua presença em cursos de água é devida, principalmente, a aplicações negligentes, como pulverizações perto dos mesmos e à escorrência dos solos. No entanto, verifica-se uma grave lacuna no que diz respeito à informação científica referente ao seu impacte genotóxico. Deste modo, a presente tese visou avaliar o risco genotóxico em peixes de duas formulações comerciais com capacidade biocida: Decis®, um inseticida que tem a deltametrina como base da sua composição química, e Roundup®, um herbicida, com glifosato como princípio ativo, representando desta forma duas classes de pesticidas com vasta utilização. Concretamente, o presente estudo delineou-se de forma a responder aos seguintes objetivos: (i) a avaliação do potencial de indução de lesões cromossómicas pelo Decis®, (ii) a capacidade do Roundup® na indução de dano no ADN, (iii) o envolvimento de processos de dano oxidativo na perda de integridade do ADN assim como (iv) a participação dos processos de reparação do ADN na progressão do dano induzido pelo Roundup®.

Adotou-se a enguia europeia (*Anguilla Anguilla* L.) como organismo-teste, submetendo-a a concentrações ambientalmente realistas de Decis® e Roundup® durante exposições de curta duração (3 dias), seguidas por avaliações de pós-exposição de 1, 7 e 14 dias como forma de avaliar a evolução do dano após a cessação da exposição.

O dano genotóxico induzido pelo Decis® foi avaliado adotando o teste das anomalias nucleares eritrocíticas (ANE), através do qual se podem observar lesões cromossómicas sinalizando um tipo de dano de difícil reparação. A genotoxicidade do Roundup® foi determinada utilizando o ensaio do cometa que deteta quebras no ADN, sendo este tipo de dano suscetível de ser reparado. Com o intuito de esclarecer o envolvimento de processos oxidativos na genotoxicidade deste herbicida, o ensaio do cometa foi melhorado com uma etapa adicional, através da qual a incubação com enzimas de reparação, as endonucleases FPG e EndoIII, permite a deteção de purinas e pirimidinas oxidadas, respetivamente. A aplicação do ensaio da reparação por excisão de bases (REB) permitiu avaliar a capacidade de reparação de dano oxidativo no ADN em enguias expostas *in vivo* a Roundup®.

No geral, os pesticidas Decis® e Roundup® mostraram ser genotóxicos para *A. anguilla*, tendo em conta a deteção de dano citogenético e no ADN, respetivamente. Além disso, em ambos os casos a genotoxicidade mostrou ser de natureza temporária, uma vez que os peixes demonstraram a capacidade de reverter completamente as lesões previamente induzidas. A exposição ao inseticida Decis® levou ao aumento da frequência de ANE, como resultado das suas propriedades clastogénica e/ou aneugénica. Este parâmetro assumiu níveis semelhantes aos medidos nos indivíduos do grupo controlo 7 dias após a cessação da exposição ao contaminante. A recuperação do dano citogenético parece resultar de uma remoção preferencial das células com morfologia nuclear anómala, tendo em conta que não se observaram alterações na dinâmica eritropoética.

O herbicida Roundup® confirmou a sua capacidade de induzir dano no ADN de células hepáticas e, apesar de não se refletir num aumento de quebras resultantes dos sítios sensíveis à FPG e à EndoIII, o envolvimento dos processos oxidativos foi confirmado. O dano no ADN de enguias expostas a este agroquímico deixou de ser percetível 1 dia após a cessação da exposição, demonstrando ser um tipo de dano de fácil reparação.

As enzimas de reparação de ADN revelaram ser vulneráveis a pressões inibitórias associadas a elevados níveis de Roundup® (constituintes e/ou metabolitos) e/ou espécies reativas de oxigénio (ERO) que provavelmente ocorrem no fígado durante o período de exposição. Contudo, no período de pós-exposição, observou-se um aumento da reparação do dano oxidativo no ADN, tornando-se deste modo, uma importante via para a recuperação completa do dano genético.

No que diz respeito às metodologias adotadas na concretização da presente tese (teste das ANE e técnica do cometa), ambas revelaram ser adequadas enquanto ferramentas de deteção dos tipos de dano descritos, confirmando a relevância da sua aplicação na avaliação do risco genotóxico de contaminantes em peixes. Tendo em conta a especificidade dos tipos de dano genético associados a cada uma das técnicas, a sua utilização complementar deverá ser considerada como uma mais-valia em diferentes momentos, considerando períodos de exposição/pós-exposição.

Globalmente, os resultados obtidos através deste trabalho indicaram que as comunidades aquáticas expostas a pesticidas se encontram sob risco, no que concerne à sua integridade genómica. Neste sentido, os dados obtidos apontam ainda para a necessidade de incrementar o número de estudos de avaliação do risco ambiental para as ictiopopulações. Deve ainda ser investigado o impacte de exposições curtas de pesticidas, tendo em conta a capacidade dos peixes recuperarem rapidamente dos efeitos causados a curto prazo. Neste sentido, é importante estabelecer medidas rigorosas que apontem para a diminuição da utilização de pesticidas e de comportamentos negligentes, assim como conduzir à escolha de compostos menos tóxicos.

keywords

Pesticides, aquatic contamination, *Anguilla anguilla*, Decis®, Roundup®, genotoxicity, DNA repair.

abstract

The presence of pesticides in water bodies is a worrying environmental issue, occurring mainly due to spray-drift, surface runoff or inadvertent applications. This vast class of agrochemicals is known to induce several pernicious effects in non-target aquatic organisms, namely fish. However, data concerning the genotoxic impact of these compounds are scarce. Hence, the present thesis aimed to fill the knowledge lacuna on pesticides capacity to induce genotoxicity to fish, addressing two commercial formulations: Decis®, a deltamethrin-based insecticide, and Roundup®, a glyphosate-based herbicide, each representing a widely used class of biocides. The present work aimed to assess: (i) the chromosomal damaging potential of Decis®, (ii) the DNA damage induction by Roundup®, (iii) the involvement of oxidative processes on the DNA integrity loss as well as (iv) the involvement of DNA repair system in the progression of the DNA damage induced by Roundup®.

European eel (*Anguilla anguilla* L.) was adopted as test organism, performing short-term exposures of 3 days to environmentally realistic concentrations of Decis® and Roundup® and post-exposure evaluations of 1, 7 and 14 days to assess the damage evolution in pesticide-free water.

In order to assess the genotoxic damage induced by Decis®, the erythrocytic nuclear abnormalities (ENA) assay was performed, evaluating chromosomal damage, a hardly repairable type of lesion. In what concerns to Roundup® experiment, the comet assay was adopted as genotoxic endpoint, measuring DNA damage as strand breaks, able to be repaired. In order to assess the involvement of oxidative damage in Roundup®-induced genotoxicity, the comet assay was performed with an extra step, where nucleoids were digested with the endonucleases formamidopyrimidine DNA glycosylase (FPG) and endonuclease III (EndoIII), measuring oxidized purines and pyrimidines, respectively. The base excision repair (BER) assay was adopted to evaluate the oxidative DNA damage repair ability of eels exposed *in vivo* to Roundup®.

In general, Decis® and Roundup® demonstrated their genotoxic induction to *A. anguilla*, since they induced cytogenetic and DNA damage, respectively. In both cases, the damage showed to be of transient nature, since fish were able to completely reverse the previously induced damage. Decis® demonstrated its genotoxic potential by the increase of erythrocytic nuclear abnormalities frequency as a result of its clastogenicity and/or aneugenicity properties. Cytogenetic damage values of exposed eels returned to control levels after 7 days in insecticide-free water. This recovery appeared to be mainly a result from a preferential removal of cells with abnormal nuclear morphology, since no alterations were noticed at the erythropoiesis dynamics.

Roundup® was capable of inducing DNA damage in hepatic cells and, though not directly reflected in an increase of breaks at FPG- and EndoIII-sensitive sites, the involvement of oxidative processes in Roundup® genotoxicity was confirmed. The DNA damage values of exposed eels returned to control levels after 1 day in herbicide-free water, mainly due to the repairable condition of this type of damage and the cessation of the exposure.

DNA repair enzymes seem to be susceptible to inhibitory actions associated to higher levels of Roundup® constituents/metabolites and/or ROS likely to occur in liver during the exposure period. However, in the post-exposure period, an increased capacity to repair oxidative DNA damage emerged, being a crucial pathway for the complete recovery from the genetic damage induced.

Evaluating the performance of the genotoxic endpoints adopted in the present study, ENA and comet assays revealed to be suitable to detect the described types of damage. In addition, it became clear that both should be used as complementary tools, since each one measures specific damage types that might be detected at different moments concerning exposure/post-exposure periods.

In what concerns the management/monitoring practices of pesticides use, the present thesis demonstrated that aquatic biota is under substantial threat and provided useful data to develop stricter regulatory directives. Further studies on this subject should be encouraged, contributing to the increased knowledge and the assessment of the environmental risk to fish populations. In addition, it should be investigated the real impact of pesticides, considering the fish ability to rapidly reverse the short-term effects. This might help the establishment of stricter regulatory procedures aiming the reduction of pesticides overuse and/or inadvertent applications as well as the choice of less toxic compounds.

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Chapter 1

General Introduction

1. General introduction

1.1 Pesticides and their environmental impact

Environmental contamination by toxic compounds has been associated to human presence and actions, resulting in a general associated risk. Among those innumerous compounds, it can be named agrochemicals, metals, biological agents (Blasco and Picó 2009).

Along with the agriculture evolution, the need of controlling pests and improving the cultures resulted in the discovery and development of a remarkable variety of agrochemicals. Pesticides, defined as any substance or mixture of substances that are intended to prevent, destroy, repel or mitigate any pest (U S EPA 2013a), are a major group of pollutants, which ubiquitous presence in the environment is due to the wide utilization and the accidental or deliberated releases. This group of chemicals are, by design, toxic to certain life forms, making it possible to control harmful organisms in diverse sectors as agriculture, forestry, industry and public health (Aprea et al. 2002; Levine 2007).

Rachel Carson' Silent Spring (1962) marked an important step to environmental awareness, warning about the excessive use of pesticides. From this point on, several organizations as the US Environmental Protection Agency (US EPA) start to develop a stricter regulation concerning agrochemicals use, as well as studies addressing human and environmental risks (Taylor et al. 2007).

Nowadays, the number of registered products with pesticide properties amounts to almost 1300 active substances in Europe (EC 2012) and more than 800 in the USA (Levine 2007). Considering the large variety of these biocides, it is possible to divide them into several classes according to the target organism against which they are used: insecticides, herbicides, rodenticides, fungicides, nematicides and others (Levine 2007). US EPA revealed that herbicides are the most used group worldwide (40%), followed by insecticides (29%), fungicides (22%) and others (9%) (Grube et al. 2011). Pesticides can also be classified according to its chemistry (eg. organochlorines, organophosphates, carbamates, pyrethroids, among others) or active principle origin (natural or synthetic) (Fernando and Duarte 2011; Levine 2007).

Although the undeniable advantages of pesticides utilization – mainly the improvement of crops production, but also, human, domestic animals and cattle welfare – potential human and environmental risks should always be evaluated alongside.

Furthermore, pesticides not always present the expected environmental fate, contradicting the presupposition of an ideal pesticide, which should be transformed into harmless materials by biological, chemical or photochemical degradation. Additionally, the resistance that some of the pests developed to the pesticides is another disadvantage to take into account (Levine 2007).

1.2 Pesticides as water pollutants

In 2000, the European Commission (EC) and all European Union (EU) member states established a Water Framework Directive, which main goals reside in achieving cleaner and safer surface and ground waters for all citizens, with their own involvement in water quality protection/management and addressing the urban waste waters and agriculture as the most important source of pollution (EC 2012).

Regarding agricultural activities, pesticides represent one of the most complex water quality problems as well as the one with lower level of knowledge about (Rickert 1993; Schwarzenbach et al. 2006). The agrochemicals that are not degraded or absorbed by plants or adsorbed by soil, possibly will enter the aquatic environment due to inadvertent applications, surface run-off and soil leach, affecting thus surface and ground waters. Additionally, particles of pesticides resulted from spray-drift applications can be carried by wind over very long distances (NPTN 2000; Ongley 1996; Pimentel 2005).

A pesticide is commonly constituted by the active and “inert” ingredients, being the latter added to the formulation to increase the effectiveness of the former. However, the designation “inert” does not necessarily mean that it is a non-toxic substance (U S EPA 2013b). Therefore, the impact of pesticides on water quality is directly related with its formulation, including active and “inert” ingredients, as well as the associated impurities and the degradation products (Ongley 1996).

The ecological risk of a pesticide (as a substance or mixture of substances) is directly related with several criteria, including the response of an organism (acute or chronic), the environmental persistence (measured as the ingredients half-lives) and the environmental degradation process and fate (Ongley 1996). Thus, the impact of a pesticide in water ecosystems is evaluated according to those criteria, including its toxicity to non-target organisms. Once these products enter the aquatic environment, their presence pose a potential risk to human health and to the aquatic organisms, namely fish, that might end up particularly affected. Fish are particularly endangered due to the forms of exposure to the aquatic contaminant: dermal contact, breathing and water ingestion. The consequences to fish are variable for each pesticide and even at sub-lethal

concentrations, the chronic effects may include tumors, reproduction impairment, deficiency on immune system as well as cellular and DNA damage (see point 1.3) (Ongley 1996).

1.2.1 Decis® and Roundup® - cases of study

Deltamethrin (Fig. 1), a non-selective insecticide, is a synthetic pyrethroid widely used to protect several fruit and vegetable crops, that acts by contact (Velíšek et al. 2007; WHO 1990). Deltamethrin was quantified in the aquatic environment at concentrations between 0.001 and 0.043 μgL^{-1} (Elfman et al. 2011) and a previous study reported values of 0.1 μgL^{-1} (Lidstone 1987). Decis®, distributed by Bayer CropScience, is the most used commercial formulation containing deltamethrin as the active ingredient, and calcium 4-(4,6,8-trimethylnonan-3-yl)benzenesulfonate, 2-methyl-1-propanol and naphtha (petroleum) as “inert” ingredients.

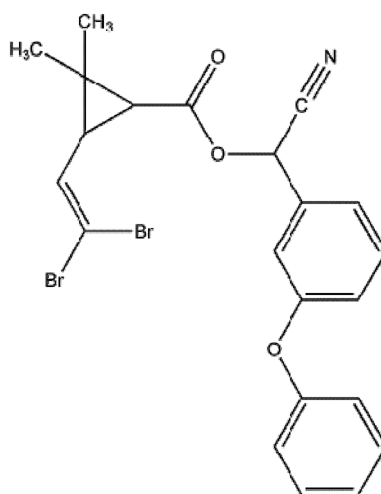

Figure 1 – Chemical structure of deltamethrin (Source: Kegley et al. 2011).

The commercial formulations containing deltamethrin, as Decis®, work as neuropoisons acting on the axons in the peripheral and central nervous systems (by interacting with sodium channels in mammals and/or insects). The synthetic pyrethroids as deltamethrin have been described as the most toxic group of insecticides to fish and aquatic invertebrates (Helfrich et al. 2009), although no serious adverse effects have been noticed due to the punctual applications to cultures and lack of persistence in the environment (WHO 1990).

Several studies evaluating the sublethal toxicity of the commercial formulation Decis® and deltamethrin on fish showed that they are capable of inducing several deleterious

effects *viz.* structural and histological changes (Al-Ghanbousi et al. 2012; Cengiz 2006; Cengiz and Unlu 2006), biochemical and hematological alterations (Hernández-Moreno et al. 2010; Svobodová et al. 2003; Velíšek et al. 2007), as well as genotoxic damage (see point 1.3.4) (Ansari et al. 2009; Grisolia 2002).

Glyphosate (Fig. 2) is a post-emergent, systemic and non-selective herbicide, used on both agricultural and non-agricultural purposes, widely used due to its efficacy and low cost (Monheit 2007). Glyphosate was quantified at surface waters in a range between 0.01 and 0.7 mgL^{-1} (Peruzzo et al. 2008), reaching 1.7 $mgL^{-}1$ after a direct application in aquatic systems (Horner 1990). The most used commercial formulation is Roundup®, distributed by Bayer Cropscience, in which glyphosate is formulated as the isopropylamine salt, also containing polyethoxylated amine (POEA) as a surfactant.

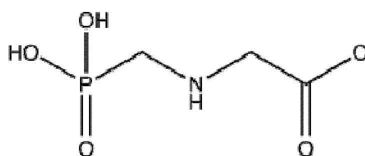

Figure 2 – Chemical structure of glyphosate (Source: Kegley et al. 2011).

Glyphosate is absorbed in plant leaves and acts by inhibiting the enzyme enolpyruvylshikimate phosphate synthase, responsible for the biosynthesis of chorismate intermediate in the biosynthesis pathways of the essential amino acids tryptophan, tyrosine, and phenylalanine, thus inhibiting the plant growth (Williams et al. 2000). Considering that this biochemical pathway is inexistent in animals, glyphosate was supposed to be harmless to animal species (Monheit 2007).

Glyphosate-based products already demonstrated its ability to inflict toxic effects in fish, leading to several physiological and biochemical alterations, such as inhibition of antioxidant capacity and induction of oxidative stress (Lushchak et al. 2009; de Menezes et al. 2011; Modesto and Martinez 2010), as well as reproductive impairment (Harayashiki et al. 2013), histological changes (Langiano and Martinez 2008) and genotoxic damage (Cavalcante et al. 2008; Grisolia 2002; Guilherme et al. 2010) in different tissues of several species.

1.3 DNA as a target for pesticides

The integrity of the DNA molecule is crucial to the health and survival of the cell. However, it is frequently a target from endogenous and exogenous sources of genotoxic stress. Endogenous attacks may occur due to the presence of substances present in the cells, such as reactive oxygen species (ROS) produced under conditions of stress, besides the basal metabolic activity. In what concerns to the exogenous agents, all the compounds present in the environment with genotoxic ability have the potential to alter the structural integrity of the DNA molecule (Geacintov and Broyde 2010). Among those compounds, pesticides have been studied and described as genotoxicants (Bolognesi 2003; Bull et al. 2006; Singh et al. 2011), including herbicides (Zeljezic et al. 2006), insecticides (Ündeğer and Başaran 2005) and fungicides (Grisolia 2002).

1.3.1 General mechanisms of genotoxicity

Bearing in mind the importance of an incorrupted DNA, the future of the cell (and its future generations) might be compromised when an exposure to a genotoxic stress takes place, since it may lead to protein dysfunction, clastogenesis and/or oncogene activation (Shugart and Theodorakis 1996). Therefore, when a compound is able to interfere with the DNA molecule, the resulting damage can include different types of genetic lesions, such as base modification, DNA adducts, DNA single- and double-strand breaks and chromosomal aberrations.

DNA bases can be modified by several mechanisms, including alkylation, deamination and oxidation, through which the genotoxicants can covalently bind and alter the structure of the DNA nucleotides. Thus, DNA adducts, for instance, result from the covalent binding of certain chemicals. This susceptibility is explained by the presence of nucleophilic sites in the DNA (negative charged sites) that are easily attacked by electrophilic (positive charged) substances, resulting in a diverse set of DNA modified bases (Tretyakova et al. 2012). Moreover, bases can be attacked by ROS that are extremely reactive molecules containing oxygen and unpaired electrons (e.g. $OH^{\bullet}$) that might affect the DNA deoxyribosyl backbone molecule, resulting in base modifications or single- and double-strand breaks, possibly resulting in promutagenic lesions (Valko et al. 2004; Weaver 2008).

DNA breaks can result either from the attack of chemical or physical agents as well from errors at the DNA replication and repair processes (Negritto 2010). Single strand-breaks are not particularly serious to the DNA molecule, since they can be easily repaired. On the other hand, double strand-breaks are probably the most deleterious type of DNA

damage, since they are, actually, broken chromosomes that if not repaired, can lead to cell death or to cancer cells (Weaver 2008). Clastogenic agents can also induce alterations at the chromosome structures, resulting in chromosomal aberrations, including ring chromosomes, chromatid interchanges and chromosomal fragments (Pfeiffer et al. 2000). Clastogenicity along with aneugenicity (a defective mitotic spindle, kinetochore or other parts of the mitotic apparatus, leading to a faulty chromosomal segregation during anaphase) are the main causes of nuclear abnormalities presented by some cells, including micronuclei. Thus, nuclear buds contain genetic material that was separated and dislocated from the original nucleus (Fenech 2000; Stoiber et al. 2004). Structural chromosomal aberrations may also be a result of replication of a DNA damaged molecule, DNA synthesis inhibition and other mechanisms as topoisomerase inhibition (Albertini et al. 2000; Mateuca et al. 2006).

1.3.2 Methodologies for the genetic damage evaluation

The adoption of genotoxic endpoints intends to assess the relation between the exposure to a genotoxicant and the resulting effects at the individual level. These methodologies are currently understood as suitable tools to evaluate the level of contamination of a certain environment, being considered useful biomarkers of environmental risk assessment (Ohe et al. 2004; Scalon et al. 2010).

(i) The erythrocytic nuclear abnormalities (ENA) assay

The ENA assay is a standard method applied to organisms with nucleated erythrocytes to assess cytogenetic damage due to the genotoxicant ability to induce chromosomal damage. The genotoxins can induce structural chromosomic alterations by cleavage (clastogenicity) or even the total loss of the chromosome and by mitotic spindle apparatus dysfunction (aneugenicity) (Fenech 2000; Stoiber et al. 2004). Thus, mature erythrocytes with micronuclei and other nuclear abnormalities are detected (Pacheco and Santos 1997). Although the establishment of the abnormality categories is not consensual, five nuclear lesions can be considered: kidney shaped nuclei (K), lobed nuclei (L), binucleate or segmented nuclei (S), micronuclei (MN) and notched nuclei (N), (Guilherme et al. 2008; Marques et al. 2009; Pacheco et al. 2005; Pacheco and Santos 1998) as illustrated at Figure 3. The determination of those nuclear abnormalities results in a frequency corresponding to the number of abnormal nuclei per 1000 cells.

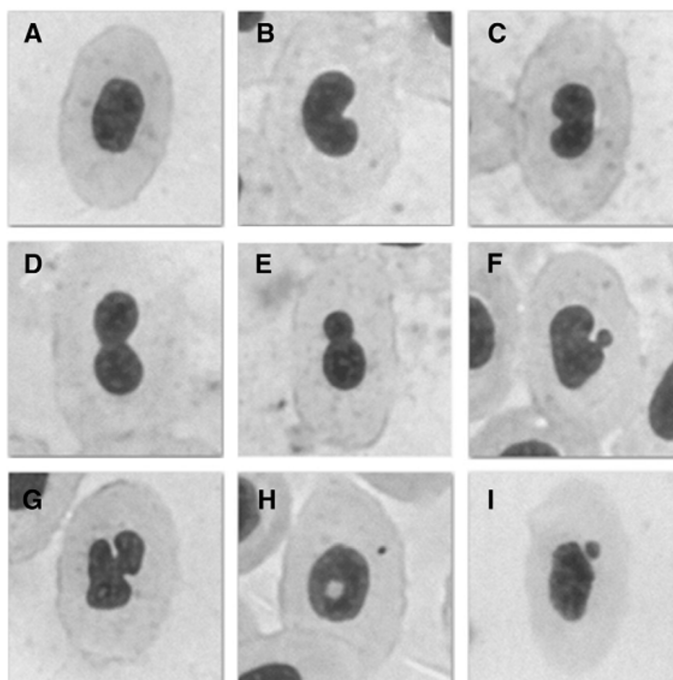

Figure 3 – Mature fish erythrocytes showing a normal nucleus (A) and nuclear abnormalities: kidney shaped (B and C), segmented (D and E), lobed (F and G), notched (H) and micronuclei (I) (Adapted from: Silva et al. 2011).

The ENA assay has been considered a suitable method to evaluate the genotoxicity of pesticides to fish, including insecticides (Ansari et al. 2009; Grisolia 2002; De Marco et al. 2000) and herbicides (Cavas 2011; Grisolia 2002; Guilherme et al. 2010).

(ii) The comet assay

The alkaline single cell gel comet assay is a standard method used to measure DNA strand breaks, as well as other type of DNA lesions (pyrimidine dimers, oxidized bases, alkylation damage). This technique relies on the principle that a lised cell embedded in agarose shows its nucleoids, including supercoiled DNA, RNA and associated proteins (Collins 2004). After an unwinding step and an electrophoresis, the single-strand DNA migrates throughout the agarose, revealing the amount of breaks. This assay is capable of showing each cell as a comet shaped nucleoid, with head and tail. The characteristics of the tail and head reveal the intensity of the DNA damage, i.e., if a cell presents a concentric nucleoid, with no tail, it has no DNA damage. On the other hand, if a cell presents almost no DNA in the head and a long tail, this is a cell with severe DNA damage (Collins 2004). Thus, the comets are classified into damage classes from 0 (no tail) to 4

(almost all DNA in tail), as can be observed in Figure 4 (Azqueta et al. 2009; Collins 2004).

Figure 4 – Images of comets stained with ethidium bromide, elucidating the visual scoring classification from 0 (no tail) to 4 (almost all DNA in tail) (Adapted from: Kleinsasser et al. 2004).

The use of comet assay standard procedure to measure DNA strand breaks is often considered as limited, since this type of DNA damage can be quickly repaired, or a result of breaks at alkali labile sites (Lee and Steinert 2003; Speit and Schütz 2008). Thus, in order to improve its sensitivity and to shed light on the eventual oxidative cause in the observed damage, the comet assay is frequently used with an extra step where nucleoids are digested with enzymes that recognize, in particular, the oxidative DNA damage, creating a break. Among the lesion specific enzymes, endonuclease III (EndoIII) and formamidopyrimidine DNA glycosylase (FPG) are often used. EndoIII detects oxidized pyrimidines while FPG allows the detection of the major purine oxidation product 8-oxoguanine. As mentioned above, and in both cases, the enzyme-sensitive sites are converted into additional breaks, resulting in an increase of the tail intensity (Azqueta et al. 2009; Collins 2004; Collins et al. 1993; Dusinská and Collins 1996).

The comet assay is a genotoxicity assessment tool considered to be valuable and suitable to adopt in environmental risk studies concerning aquatic organisms (Frenzilli et al. 2009; Ohe et al. 2004; Scalon et al. 2010). The comet assay has been widely used in genotoxic potential evaluation concerning fish exposure to pesticides, including herbicides as Roundup® (Cavalcante et al. 2008; Cavas 2011; Guilherme et al. 2012b; Guilherme et al. 2010).

Since the comet and the ENA assays allow the detection of different types of genetic damage, DNA strand-breaks and chromosomal abnormalities respectively, both are able to provide independent and significant data. Thus, the comet and the ENA assays should be considered and adopted as complementary genotoxic endpoint tools (Wirzinger et al. 2007).

1.3.3 DNA repair

The exposure to a genotoxicant itself might not be enough to provoke severe genetic damage. In fact, the cell has a set of repair systems that allow the balance between DNA lesions and DNA integrity. Thus, one factor that is important to take into account is the DNA repair ability of each cell. DNA primary damage (DNA adducts, DNA strand-breaks) is susceptible to be tolerated or repaired. However, the tolerance of the error or the faulty repair, might lead to the replication and perpetuation of the damage and possibly ends severely affecting the cell by protein dysfunction, clastogenesis and oncogene activation (Friedberg 2003; Shugart and Theodorakis 1996).

There are various types of mechanisms removing DNA lesions, namely single- and double-strand breaks repair, mismatch repair, nucleotide and base excision repair, among others. There are two basic ways to deal with DNA damage: directly undo the damage (e.g. photoreactivation repair) or remove DNA damaged sections (excision repair). Basically, damaged DNA is removed and replaced with new, undamaged DNA, by one of two mechanisms: nucleotide excision repair (NER) or base excision repair (BER). The first one generally deals with severe DNA lesions, recognizing the strand with damage, cutting on either side of it, removing an oligonucleotide (24-32 nucleotides). The removal is followed by DNA polymerase activity, which fills the gap and the nick is sealed by a DNA ligase (Weaver 2008).

In what concerns to BER, this mechanism appears as the most prevalent and removes common and subtle changes to DNA bases, being in general similar to the NER. Thus, the damaged base is recognized and removed by an enzyme called DNA glycosylase, leading to a break in the glycosidic bond between the sugar and the damaged base which will result in an apurinic/apyrimidinic (AP) site. Afterwards, an AP endonuclease completes the break which will be filled with a new base by a DNA polymerase. Finally, the nick is sealed by a DNA ligase, repairing the DNA molecule (Weaver 2008).

1.3.4 Genotoxicity and DNA repair in fish – Decis® and Roundup® cases of study

Bearing in mind the information mentioned above concerning pesticides threat to non-target organisms, the studies addressing genotoxic potential of the insecticide Decis® are scarce. Grisolia (2002) pointed out a higher sensitivity of fish *Tilapia rendalli* to Decis®, in comparison to mouse, showing an increase of the micronuclei frequency after the exposure to this commercial insecticide. Similarly, Ansari et al. (2009), revealed the

induction of micronuclei and other nuclear abnormalities after short-term exposures of the fish *Channa punctata* to deltamethrin (Decis® active ingredient).

Concerning the genotoxicity induced by the herbicide Roundup®, this subject is better documented, since several studies confirmed its genotoxic potential. Grisolia (2002) tested this commercial formulation and assessed the induction of MN in fish *T. rendalli*, pointing out its higher sensitivity to Roundup® in comparison to mice. Çavas and Könen (2007) evaluated the effects of Roundup® in fish *Carassius auratus*, showing an increase of micronuclei and other nuclear abnormalities, as well as DNA strand breaks. In a similar study, Cavalcante et al. (2008) confirmed the genotoxic potential of Roundup®, since DNA strand breaks (measured by the comet assay) were significantly higher in exposed fish *Prochilodus lineatus*. Guilherme and co-workers (2010; 2012a; 2012b) studied this subject under different approaches, generally concluding that Roundup® is genotoxic to the fish *Anguilla anguilla* at realistic concentrations and after short-term exposures, revealing that this herbicide induces erythrocytic nuclear abnormalities in blood, as well DNA strand breaks in blood, liver and gills. Recently, Ghisi and Cestari (2013) confirmed the genotoxic ability of this herbicide by the comet assay in blood and hepatic cells of fish *Corydoras paleatus*. Concerning the genotoxicity mechanisms of Roundup®, Guilherme et al. (2012a) suggested the oxidative stress as a possible mechanism involved on DNA damage.

In what concerns to the evaluation of DNA repair ability in fish exposed to Roundup®, to author's knowledge there are no studies considering this matter, which can be pointed out as a significant lacuna concerning genotoxic risk. In a recent review, Kienzler et al. (2013) defended this endpoint as a very significant tool in ecotoxicology studies, since DNA repair is the first defense line against genotoxicants and passible of being also affected by them.

Therefore, concerning the wide utilization of the pesticides mentioned above as well as their input in the water systems, the importance of evaluating their genotoxic ability in aquatic organisms should be emphasized.

1.4 Goals and thesis structure

Taking into account the information concerning the genotoxic risk of pesticides to fish, this work aimed to fulfill various knowledge gaps. Thus, the main goals were: (i) to evaluate the genotoxic potential of the pesticides Decis® and Roundup® after a short-term exposure, as well as the evolution of the genetic damage during the post-exposure; (ii) to clarify the mechanisms involved in their DNA damage ability; (iii) to contribute to the

improvement of monitoring and managing actions concerning forestry and agriculture practices, mitigating the effects of agrochemicals in aquatic biota.

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

- Assessment of the chromosomal damage induction by Decis® insecticide by the ENA assay
- Evaluation of the DNA damage induction by Roundup® herbicide by the comet assay
- Clarification of the involvement of oxidative damage on the DNA integrity loss induced by Roundup®, through the comet assay improved with the adoption of the DNA lesion-specific enzymes FPG and EndoIII
- Assessment of the oxidative DNA damage repair processes in the progression of DNA damage induced by Roundup® by the BER assay

The study was carried out using the European eel (*Anguilla anguilla*), adopting exposure experiments of 3 days and post-exposure of 1, 7 and 14 days.

The present thesis comprises four chapters (1-4), in which is included a general introduction (Chapter 1) addressing the necessary information related to the following chapters 2 (assessment of Decis® genotoxicity) and 3 (evaluation of the genotoxic potential of Roundup® and its modulation of the DNA repair system) and Chapter 4, including the final remarks of the present study.

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Chapter 2

Induction and recovery of cytogenetic damage in peripheral erythrocytes of fish (*Anguilla anguilla*) upon exposure to a deltamethrin-based insecticide

2. Induction and recovery of cytogenetic damage in peripheral erythrocytes of fish (*Anguilla anguilla*) upon exposure to a deltamethrin-based insecticide

Abstract

The pyrethroid insecticide Decis®, containing deltamethrin as active ingredient, is among the most popular broad-spectrum biocides, with wide application in agriculture and home pest control. The occurrence of deltamethrin in the aquatic environment is well-established, but the possible genotoxic effects of Decis® in non-target organisms, namely fish, remain unknown. Hence, this work aimed to evaluate the cytogenetic damaging potential of Decis® in European eel (*Anguilla anguilla*), adopting the erythrocytic nuclear abnormalities (ENAs) assay. In addition, it was intended to investigate the damage progression in the post-exposure period. The frequency of immature erythrocytes (IE) was determined to provide indirect information on the erythrocyte catabolism and erythropoiesis rate. Fish were exposed to 17.5 and 35 μgL^{-1} of Decis® (equivalent to 0.05 and 0.1 μgL^{-1} of deltamethrin, respectively) during 1 and 3 days. Thereafter, fish were transferred to insecticide-free water and kept for 1, 7 and 14 days. The results demonstrated a clear potential to induce chromosomal damage following 3 days exposure, depicted in an ENA frequency increase for both Decis® concentrations. The transient nature of this cytogenetic damage was also demonstrated, as ENA frequency returned to the control level 1 and 7 days after cessation of the exposure, respectively for the higher and the lower Decis® concentration. Moreover, this response pattern provided evidence towards a rapid metabolization and elimination of the constituents of the tested formulation by *A. anguilla*, namely deltamethrin. Overall, the demonstrated genotoxic properties of Decis® pointed out increased risk factors to fish exposed to this insecticide.

Keywords

Decis®; genotoxicity; ENA assay; chromosomal damage recovery; fish

2.1 Introduction

The use of biocides to control harmful organisms is regarded as an unavoidable tool in several sectors, namely agriculture, forestry, industry and public health. Since they are intended to kill living organisms, numerous biocidal products have also an inherent capacity to cause adverse effects on humans or the environment. In this direction, it is of particular concern the contamination of aquatic systems, as these compounds are easily washed from surfaces, mainly due to natural processes as water runoff and soil leaching, reaching superficial waters also by spray-drift during applications. Therefore, it is undeniable that aquatic organisms, namely fish, will be exposed by dermal contact, breathing and water ingestion, ending particularly affected (Helfrich et al. 2009; Pimentel 2005).

Among the large variety of biocides, defined according to organism against which are used, insecticides have triggered a considerable environmental apprehension due to their high toxicity extensively demonstrated in non-target organisms (Aprea et al. 2002), including fish (Clasen et al. 2012; Moreira et al. 2010; Parent et al. 2011). Pyrethroids are among the most used insecticides worldwide and one of the most toxic group to fish and aquatic invertebrates (Helfrich et al. 2009), which has been explained by their lipophilicity and high rate of gill absorption (Demoute 1989; Viran et al. 2003). Nonetheless, it has been also stated that the low doses commonly applied and the low persistence in the environment can avoid the major adverse effects for those organisms (WHO, 1990). Deltamethrin, the active ingredient of the commercial formulation Decis®, is a synthetic pyrethroid with insecticidal properties, used to protect several fruit and vegetable crops, effective against a multiplicity of pests, which acts in the peripheral and central nervous systems of insects, interfering with the sodium channels (WHO, 1990). Deltamethrin has very low water solubility, strongly adsorbs to soil and sediments and degrades quickly in the environment (WHO, 1990). However, the deltamethrin-based commercial products, like Decis®, behave differently. Decis® is miscible with water, due to the surfactant, which potentially increases the bioavailability of the active principle in the dissolved phase. Hence, the presence of deltamethrin in the aquatic environment was determined in water streams nearby rice cultivations on the island of Leyte (Philippines) in concentrations between 0.001 and 0.043 μgL^{-1} (Elfman et al. 2011). Additionally, a previous study reported 0.1 μgL^{-1} of deltamethrin in water samples collected near a potato field in Manitoba (Canada) (Lidstone 1987).

Several studies have been conducted evaluating the sublethal toxicity of deltamethrin and related commercial formulations to fish. Thus, Decis® showed to induce biochemical

and hematological alterations in *Poecilia reticulata* (e.g. glutathione S-transferase and lactate dehydrogenase activities increase and acetylcholinesterase inhibition, in gills, dorsal muscles and head, respectively) (Moreira et al. 2010) and *Onchorhynchus mykiss* (e.g. decreased plasma glucose, and increased erythrocyte count, hemoglobin content, hematocrit and plasma total proteins) (Velíšek et al. 2007). The same formulation also caused histopathological effects in gills of *Aphanius dispar* (Al-Ghanbousi et al. 2012), in gills and kidney of *Cyprinus carpio* (Cengiz 2006), and in liver of *Gambusia affinis* (Cengiz and Unlu 2006).

It is well established that water contaminants, such as pesticides, can attack DNA and thus, the analysis of genetic damage in aquatic organisms is considered a suitable method for evaluating their environmental hazard (Scalon et al. 2010). Nevertheless, the studies concerning the genotoxicity of Decis® or its active principle in fish remain scarce. Ansari et al. (2009) assessed the genotoxic effect of deltamethrin in *Channa punctata*, reporting the induction of erythrocytic micronuclei and other nuclear abnormalities. Additionally, Grisolia (2002) pointed out a higher vulnerability of fish to Decis®, comparing to mouse, since only fish (*Tilapia rendalli*) showed increased micronuclei frequency following exposure to this commercial insecticide.

Taking into account the most common applications of insecticides, their input to the aquatic systems is typically intermittent. Hence, and owing also to fish mobility, the exposure to this type of contaminants can be short and eventually followed by a period permanence in non-contaminated areas. Bearing this in mind, the assessment of genotoxic endpoints after removal of the contamination source appears as a crucial approach to determine the actual magnitude of risk posed by these biocides to fish. However, this perspective is absent in the majority of fish studies evaluating the genotoxicity of pesticides.

Considering the above statements and the gaps identified, it is relevant to further assess the genotoxic potential of Decis® to fish and to investigate the damage progression in the post-exposure period. Thus, the main goal of this work was to evaluate the genetic damage in *Anguilla anguilla*, subjected to a short-term exposure (1 and 3 days) to Decis®, at environmentally realistic concentrations, and the eventual recovery after fish transfer to insecticide-free water (1 to 14 days post-exposure). The cytogenetic damage was evaluated through the erythrocytic nuclear abnormalities (ENA) assay performed in peripheral erythrocytes. This methodology relies on the fact that genotoxins can promote chromosomic cleavage (clastogenicity) or even the complete loss of a chromosome, as well as dysfunction at the mitotic spindle level (aneugenicity) (Fenech

2000; Guilherme et al. 2010; Stoiber et al. 2004). Assuming that those nuclear anomalies reflect irreparable lesions, the eventual recovery of the damage would be possible mainly by processes of cell turnover (replacement of defective cells with newly generated ones), purging the blood of abnormal erythrocytes. Therefore, the frequency of immature erythrocytes (IE) was also determined to survey the hematological dynamics, obtaining indirect information on the erythrocyte catabolism and erythropoiesis rate.

2.2 Material and Methods

2.2.1 Chemicals

The commercial formulation tested - Decis® - is distributed by Bayer CropScience Portugal, containing deltamethrin as the active ingredient at 25 gL^{-1} (2.85%), calcium 4-(4,6,8-trimethylnonan-3-yl)benzenesulfonate (1-5%), 2-methyl-1-propanol (1-5%), and naphtha (petroleum) as the surfactant (>25%). All the other chemicals were obtained from the Sigma-Aldrich Chemical Company (Spain).

2.2.2 Test animals and experimental design

A. anguilla specimens with an average weight 0.25 ± 0.02g (glass eel stage) were captured at Minho river mouth, Caminha, Portugal. Eels were acclimated to the laboratory for 15 days, maintained 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 ± 1 ºC, pH 7.3 ± 0.2, nitrate 25 ± 0.5 mgL^{-1}, nitrite 0.05 ± 0.01 mgL^{-1}, ammonia 0.1 ± 0.01 mgL^{-1}, dissolved oxygen 8.1 ± 0.5 mgL^{-1}. During this period, fish were daily fed with fish roe.

The experiment (Fig. 1) was carried out in 1-L aquaria, in a semi-static mode, under the same conditions described for the acclimation period. Two animal groups were submitted to 17.5 and 35 μgL^{-1} of Decis® (equivalent to 0.05 and 0.1 μgL^{-1} of deltamethrin, respectively), during 1 and 3 days (exposure period). Two other groups were exposed for 3 days to the previously mentioned Decis® concentrations and thereafter transferred to insecticide-free water and sampled after 1, 7 and 14 days (post-exposure period). In parallel, groups of fish were kept in clean water and sampled at the same times (control groups).

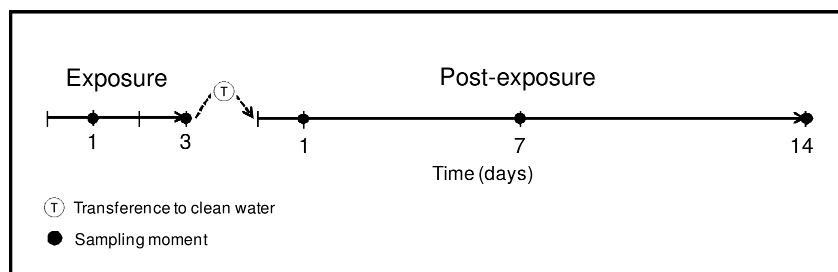

Figure 1 – Schematic representation of the experimental design, elucidating the sampling moments at 1 and 3 days (exposure period) and the transference to insecticide-free water after 3 days of exposure, with sampling moments at 1, 7 and 14 days (post-exposure period).

Each experimental group corresponded to triplicate aquaria (n=3), and 4 fish per aquarium (i.e., 3x4 = 12 fish per condition/time). Water medium in both exposure and post-exposure periods was daily renewed (100%).

Fish were not fed during the exposure period, being daily fed with fish roe along the post-exposure period. Animals were sacrificed by cervical transaction at the post-opercular region and blood collected from the heart using heparinised capillary tubes. Blood smears were immediately prepared for ENA assay and IE scoring.

2.2.3 ENA assay

In order to evaluate the genotoxicity of Decis®, the ENA assay was performed in mature peripheral erythrocytes as described by Pacheco and Santos (1997). One blood smear per animal was fixed with methanol during 10 min and stained with Giemsa (5%) during 30 min. Slides were coded and scored blind. From each smear, 1000 erythrocytes were scored, under 1000x magnification, to evaluate the relative frequency of the following nuclear lesions: kidney shaped nuclei (K), lobed nuclei (L), binucleate or segmented nuclei (S) and micronuclei (MN). Blebbed and lobed nuclei were considered in a single category - lobed nuclei - and not differentially scored due to some ambiguity in their distinction, as suggested by Guilherme *et al.* (2008). The notched nuclei (N) were also counted as suggested by Fenech (2000) and Ayllon and Garcia-Vazquez (2001). Though the frequency (‰) of each nuclear abnormality category was individually reported, the results of ENA assay were expressed as the sum of frequencies for all the categories considered (K+L+S+N+MN).

2.2.4 IE frequency

IEs were scored for each of the 1000 erythrocytes (mature and immature) per fish. Results were presented as a frequency, resulted from the expression:

$$\text{IE frequency } (^0/_{00}) = \frac{\text{IE}}{(\text{ME+IE})} \times 1000$$

where ME = mature erythrocytes and IE = immature erythrocytes.

Mature erythrocytes can be distinguished from immature since the latter present a rounder and larger nucleus and a bluish-grey cytoplasm, as established by Hibiya (1982) and Smith (1990).

2.2.5 Statistical analysis

Statistica 8.0 software was used for statistical analysis. All data were first tested for normality and homogeneity of variance to meet statistical demands. For each sampling moment, 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. For the exposure period, a 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 the interactions between them. A similar analysis was applied to post-exposure data testing the factors post-exposure time and concentration in the precedent exposure. 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).

2.3 Results

2.3.1 ENA assay

Concerning the first day of exposure, no significant alterations were found in ENA frequency, for both concentration groups of Decis® (Fig. 2), as well as in the different lesion categories analyzed individually (Table 1). Nevertheless, after 3 days of exposure, both concentrations caused significant ENA increase in comparison with the control (Fig. 2). The analysis of each individual nuclear lesion category demonstrated that K and L frequencies were significantly higher in D1 group (17.5 μgL^{-1} of Decis®) while S frequency was significantly higher in D2 group (35 μgL^{-1} of Decis®), relatively to the control group. At this exposure time, the sub-total K+L+S+N was significantly higher in both D1 and D2 groups, comparing to the control group. After 3-day exposure, kidney shaped nuclei was the most commonly detected abnormality in both D1 (K > L > S > MN > N) and D2 (K > L > S > N > MN) groups.

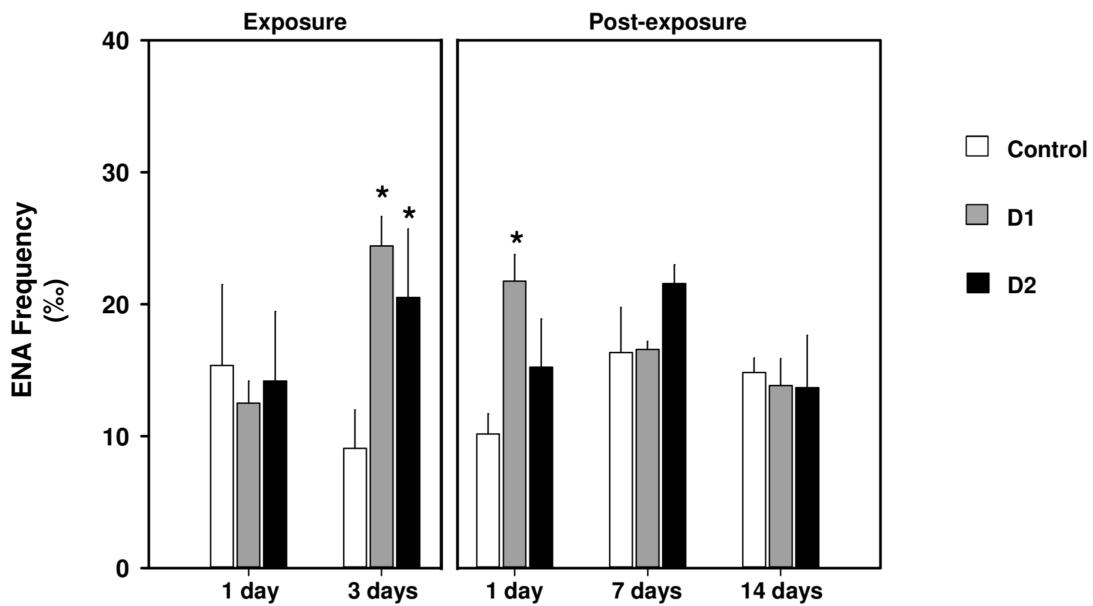

Figure 2 – Mean values of ENA frequency (‰) in peripheral erythrocytes of *A. anguilla* exposed to 17.5 (D1) and 35 µgL-1 (D2) of Decis® during 1 and 3 days and in post-exposure periods of 1, 7 and 14 days (replicate tanks, n=3). Bars represent the standard error. Statistically significant differences (p<0.05) are: * in relation to control, within the same exposure/sampling time.

In what concerns to the post-exposure period, it was observed that on day 1 only the D2 group was able to recover, since total ENA frequency decreased up to the control level (Fig. 2). The D1 group still presented significant cytogenetic damage (Fig. 2) and the kidney shape nuclei were significantly higher in this group relatively to the control, as well as the subtotal (Table 1). From the seventh day post-exposure onwards, cytogenetic damage was no more observed.

Table 1 – Mean frequency (‰) of each nuclear abnormality category (± standard error) in peripheral erythrocytes of *A. anguilla* exposed to 17.5 (D1) and 35 μgL^{-1} (D2) of Decis® during 1 and 3 days and in post-exposure periods of 1, 7 and 14 days (replicate tanks, n=3). Statistically significant differences (p<0.05) are: * in relation to control, within the same exposure/sampling time.

			Nuclear Abnormalities Categories					
			Kidney Shaped (K)	Lobed (L)	Segmented (S)	Notched (N)	Sub-total (K+L+S+N)	Micronuclei (MN)
Exposure	1 day	Control	11.53±5.14	2.83±0.93	0.75±0.52	0.00±0.00	15.11±5.98	0.25±0.14
		D1	6.83±1.71	4.25±1.15	1.17±0.36	0.25±0.14	12.50±1.66	0.00±0.00
		D2	9.25±3.38	3.50±1.38	0.92±0.55	0.42±0.22	14.08±5.22	0.08±0.08
	3 days	Control	6.25±2.18	2.08±1.08	0.67±0.36	0.08±0.08	9.08±2.91	0.00±0.00
		D1	14.97±1.14*	6.92±1.34*	1.92±0.58	0.28±0.15	24.08±2.05*	0.33±0.22
		D2	10.72±1.67	5.92±0.46	2.97±0.39*	0.53±0.12	20.14±2.31*	0.36±0.07
Post-exposure	1 day	Control	5.83±0.93	2.75±0.38	1.33±0.42	0.00±0.00	9.92±1.54	0.25±0.00
		D1	14.67±1.12*	5.17±1.02	1.83±0.30	0.00±0.00	21.67±1.97*	0.08±0.08
		D2	8.06±1.84	5.78±1.78	1.31±0.40	0.00±0.00	15.14±3.73	0.08±0.08
	7 days	Control	12.58±3.25	2.50±0.95	1.08±0.30	0.00±0.00	16.17±3.34	0.17±0.08
		D1	11.67±2.44	3.50±1.25	0.92±0.44	0.17±0.08	16.25±0.80	0.33±0.22
		D2	13.61±6.31	6.19±1.43	1.58±1.23	0.00±0.00	21.39±8.49	0.17±0.17
	14 days	Control	10.08±0.71	4.08±0.60	0.42±0.30	0.17±0.08	14.75±1.01	0.08±0.08
		D1	10.00±1.98	3.25±1.51	0.33±0.22	0.17±0.17	13.75±2.00	0.08±0.08
		D2	8.92±1.96	4.00±1.76	0.50±0.14	0.17±0.08	13.58±3.90	0.08±0.08

2.3.2 IE frequency

Though higher values were regularly found for D2 group, no significant alterations were found in IE frequency for the both exposure lengths (1 and 3 days) (Fig. 3). In what concerns to the post-exposure period, it was only observed a significant increase of the IE frequency for the group exposed to the highest concentration of Decis® (D2), relatively to the control group, on day 14 (Fig. 3).

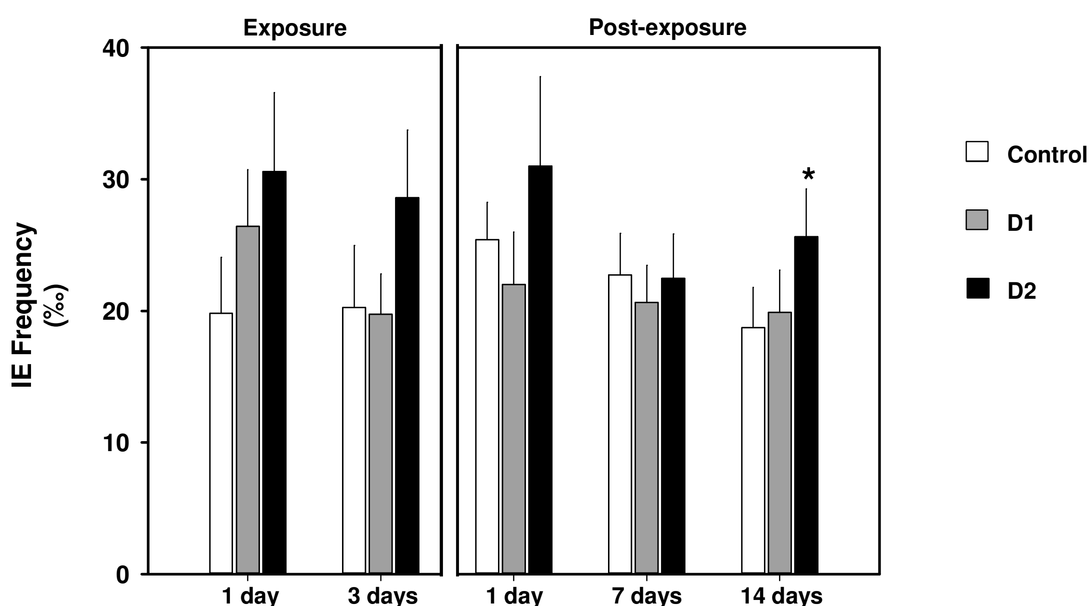

Figure 3 – Mean values of IE frequency (‰) in peripheral erythrocytes of *A. anguilla* exposed to 17.5 (D1) and 35 µgL^{-1} (D2) of Decis® during 1 and 3 days and in post-exposure periods of 1, 7 and 14 days (replicate tanks, n=3). Bars represent the standard error. Statistically significant differences (p<0.05) are: * in relation to control, within the same exposure/sampling time.

2.4 Discussion

Genotoxic effects can involve mutations and DNA damage, such as single- and double-strand breaks, which in turn can induce physical abnormalities in the chromosomes (clastogenicity). Chromosomal damage can also occur as an indirect consequence of dysfunctions at the mitotic spindle level (aneugenicity). In general, the chromosomes of fish are relatively small in size and/or high in number, making difficult the metaphase analysis of chromosomal aberrations using these organisms (Ohe et al. 2004). However, small size and large chromosome number occurring in fish do not affect the performance of the MN assay (Ohe et al. 2004) neither of the ENA assay (Pacheco and Santos 1997). Thus, both MN and ENA are widely used and well-validated cytogenetic assays for the assessment of clastogenicity and aneugenicity induced in fish by environmental genotoxicants (Fenech 2000; Stoiber et al. 2004), including pesticides (Guilherme et al. 2010).

The contamination of surface waters with genotoxic chemicals is likely to pose a serious threat to the health and survival of fish, since it can be on the basis of adverse effects such as changes in gene expression, abnormal development and cancer. However, genotoxic effects induced by pesticides in fish still remain largely unexplored.

On the other hand, since the use of deltamethrin and other pyrethroids is expected to increase in pest control, the ecotoxicologic assessment of their usage is imperative (Ansari et al. 2009). Hence, the central goal of the present study was to assess the occurrence of chromosomal damage induced by laboratory exposure to a deltamethrin-based commercial insecticide - Decis® - in peripheral erythrocytes of fish (*Anguilla anguilla*). The concentrations of Decis® currently tested correspond to 0.05 and 0.1 μgL^{-1} of deltamethrin, which should be considered environmentally realistic since this pyrethroid compound was detected in surface waters at levels up to 0.1 μgL^{-1} (Elfman et al. 2011; Pawlisz et al. 1998).

Analysing the present results, it was evident the clastogenic and/or aneugenic capacity of Decis® after 3-day exposure, since it was observed a significant increase of ENAs frequency for both concentrations. This finding is in agreement with Grisolia (2002), who also detected a positive genotoxic effect, measured through MN assay, in *Tilapia rendalli* on the fourth day following an intra-abdominal injection of the insecticide. Additionally, there is another coincidence between the response of *A. anguilla* and that of *T. rendalli* (Grisolia 2002) in what concerns to the absence of clear dose/concentration dependence.

In some extent, the present results can also be considered in line with those of Ansari et al. (2009), who found induction of MN and other nuclear abnormalities in peripheral erythrocytes of *Channa punctata* exposed to deltamethrin (0.4 - 1.2 μgL^{-1}) for 2 and 3 days. Similarly to the response pattern observed for *A. anguilla*, *C. punctata* also displayed a time-dependent response, as higher frequencies of both types of clastogenic effect were observed in fish subjected to the longer exposure (Ansari et al. 2009).

It has been demonstrated that one of the most disturbing toxic mechanisms of pyrethroids, particularly deltamethrin, in fish is the overproduction of reactive oxygen species (ROS) and subsequent induction of oxidative stress (Ansari et al. 2009; Parvez and Raisuddin 2005). It is well established that toxicants with oxidative stress potential can attack DNA, resulting in molecular and clastogenic damages (Jha 2008). In this direction, Ansari et al. (2009) suggested that oxidative stress may, in part, be contributing to the deltamethrin-induced chromosomal damage observed in fish erythrocytes. Hence, the absence of ENA increases in *A. anguilla* after 1 day exposure can be indicative of an initial protective response efficient against oxidative damage, exerted by antioxidants systems. In line with this, the subsequent occurrence of ENA induction after 3 days of exposure can be a signal of a progressive decay of cell antioxidant protection, being no longer able to counteract the negative effect of ROS on DNA. A similar explanation was

presented by Ansari et al. (2009) in relation to the induction of MN and other nuclear abnormalities accompanied by increased lipid peroxidation (LPO) in erythrocytes of *Channa punctata* exposed to deltamethrin. This interpretation was supported by the occurrence of a superoxide dismutase (SOD) activity depletion following 3-day exposure (Ansari et al. 2009).

The actual magnitude of risk posed by a given biocide depends on the balance between formation of cytogenetic damage and its loss through DNA-repair, regular cell renewal or apoptosis. Keeping this in mind, from the authors' point of view it is critical to assess the progress of the genotoxic effect in fish after removal of the contamination source, which also allows a more integrated knowledge on the mechanisms involved in the modulation of genotoxicity. Nevertheless, as highlighted in the introduction chapter, this perspective is absent in the majority of fish studies evaluating the impact of pesticides. It has been predicted that hematologic alterations (e.g. in erythrocyte and leukocyte count, hemoglobin content and hematocrit) induced by pyrethroid (cypermethrin) and carbamate pesticides in *Labeo rohita* require from 60 to 101.5 days for a complete recovery after fish transference to insecticide-free water, depending on the pesticide and parameter (Adhikari et al. 2004). To the authors' knowledge, no fish studies adopted this approach in the context of genotoxicity assessment of pyrethroid insecticides.

The present results revealed that ENA frequency returned to the control level in fish exposed to both Decis® concentrations after 7 days of permanence in insecticide-free water. Moreover, fish exposed to the higher concentration (D2) showed an earlier recover, i.e. on the first day post-exposure. These results clearly indicate a transience of the genetic damage manifestation (chromosomal damage) associated to the cessation of the exposure. An attempt to clarify the mechanisms underlying this profile of response should consider the combined involvement of some variables, *viz*. (i) the toxicokinetics of the genotoxicants, (ii) the cell turnover in the tissue under analysis, and (iii) the evolution of antigenotoxic processes like DNA-repair and antioxidant actions.

Bearing in mind the toxicokinetics of pyrethroids (i), deltamethrin included, it was demonstrated that it could be rapidly metabolized in mice and rats, mainly by the action of carboxylesterases, resulting in a fast excretion (Demoute 1989). However, it has been suggested that those enzymes are less present or less effective in fish comparing to mammals, resulting in a lower elimination rate of deltamethrin and, subsequently, explaining the fish higher sensitivity to this insecticide (Demoute 1989; Edwards et al. 1986). Nevertheless, this interpretation appears to lack scientific support since studies

characterizing carboxylesterases activity in aquatic organisms are scarce. To the authors' knowledge, the only study available in fish defended a high activity of these enzymes in the rainbow trout and its direct influence on the toxicity of pesticides (Barron et al. 1999). No studies were found evaluating the carboxylesterases activity in fish exposed to pyrethroid-based pesticides.

The half-life of deltamethrin in plasma of rats was demonstrated to be 38.5 h (Anadon et al. 1996). More recently, deltamethrin in rats showed to be almost completely eliminated from the body within 1-3 days (El-Maghraby S. 2007). The half-lives of several pyrethroids (other than deltamethrin) in rainbow trout were all greater than 48 h (Bradbury and Coats 1989). Hypothesizing a slower metabolization and elimination rates of deltamethrin in fish comparing to mammals, in the current experiment it would be expectable that *A. anguilla* still retained considerable concentrations of deltamethrin in their body 1-day after cessation of the exposure. However, ENA results (D2 group) do not support this hypothesis, pointing towards a rapid elimination of deltamethrin (and other Decis® constituents) by *A. anguilla*, strongly suggesting a metabolization and elimination rates comparable to mammals. This indication seems to be in line with the findings of Barron et al. (1999) and reinforces the importance of improving the knowledge on carboxylesterases metabolism in fish, in order to thoroughly understand the toxicokinetics and toxicodynamics of pyrethroids.

Independently of the genotoxicants toxicokinetics, the return of the ENA frequency to the control level in the post-exposure period (following a peak on third day of exposure), assuming nuclear anomalies as hardly reparable lesions, relies on the removal of erythrocytes containing abnormal nuclei and/or a dilution effect resulting from erythropoiesis (releasing new normal cells into circulation). Hence, two questions emerge in the context of the cell turnover modulation (ii) as a response to the damage induced: was the erythropoiesis rate incremented? Was the catabolism of abnormal erythrocytes improved?

To shed light in these processes, it was decided to determine the frequency of immature erythrocytes (IE) in *A. anguilla*, since it can provide indirect evidence on the erythrocyte catabolism and erythropoiesis rate. Thus, IE results suggested an unaltered erythropoiesis rate during the entire experiment, with the exception of day 14 post-exposure (where IE frequency was increased). Consequently, a dilution effect resulting from an erythropoiesis increment should be excluded as a determinant factor affecting ENA frequency in the first 7 days of the post-exposure period.

The life span of erythrocytes in fish was reported to vary among species over a range of 80-500 days (Fischer et al. 1998; Soldatov 2005). Therefore, cell removal at a basal rate would not be enough to explain the ENA frequency decline in day 1 post-exposure (for D2 group). Furthermore, if the ENA frequency reduction was explained by a basal removal rate, it would affect equally both exposed groups (D1 and D2), which did not occur. Hence, the most plausible explanation involves a preferential removal of abnormal cells in fish exposed to Decis®, which was particularly evident in D2 group. In agreement, an increased splenic erythrophagia has been previously associated with a prominent genetic damage in *A. anguilla* (Pacheco and Santos 2002). Nevertheless, the phenomenon of pronounced removal of cells with abnormal nuclear morphology may be triggered by a concomitant poor general cellular condition. Though the occurrence of metabolic disorders resulting in/directly from the DNA damage cannot be excluded as a condition leading to shortened erythrocyte survival, the loss of cell membrane integrity and stability is probably the alteration primarily responsible by the preferential removal of abnormal erythrocytes. This explanation is supported by the detection of a concentration-dependent increase of LPO in erythrocytes of fish exposed to deltamethrin for 3 days (Ansari et al. 2009). Thus, it is hypothesised that erythrocytes targeted by Decis® components undergone changes in its cell membrane (concomitantly to chromosomal damage), affecting surface deformability, flexibility, adhesion and immune recognition. This type of alterations makes erythrocytes more susceptible to burst when crossing small capillaries as well as to selective recognition by macrophages from the reticuloendothelial system (e.g. in spleen and liver).

Contrarily to chromosomal damage (e.g. after 3 days of exposure), lipid peroxidation may have occurred in a concentration-dependent manner, as demonstrated by Ansari et al. (2009) for deltamethrin. This can explain why a preferential removal of abnormal cell was perceptible in D2 and not in D1 group, on day 1 post-exposure.

On the 7th day post-exposure onwards, the absence of genotoxicants in the medium and the probable drastic reduction of the internal concentrations of deltamethrin (and other Decis® components) promoted a predominance of antigenotoxic processes (iii), namely DNA-repair and antioxidant defences, impairing the appearance of new nuclear abnormalities. The increase in the IE frequency detected on the 14th day post-exposure may be regarded as a compensatory effect to cope with a slightly higher erythrocyte catabolism observed continuously during the entire experiment (though statistically insignificant) for the higher concentration of Decis®.

Even considering that *A. anguilla* restored the chromosomal integrity in erythrocytes after 7 days of permanence in insecticide-free water, the interpretation of this response profile as a complete recovery and an abolishment of risk should be taken carefully, since the occurrence of long-term genome-destabilizing effects cannot be excluded.

2.5 Conclusions

Overall, the present findings highlighted the genetic hazard to fish associated to the deltamethrin-based insecticide Decis®, since a potential to induce chromosomal damage following a short-term exposure (3 days) was clearly demonstrated.

The transient nature of the cytogenetic damage, measured as ENA induction, was also demonstrated. Thus, ENA frequency returned to the control level 1 and 7 days after cessation of the exposure, respectively for the higher and the lower Decis® concentration. The restoration of ENA frequency in the post-exposure period appeared to involve a preferential removal of cells with abnormal nuclear morphology, since the balance erythropoiesis/erythrocyte catabolism was not altered as depicted in the stable IE frequency. This response pattern provided evidence towards a rapid metabolization and elimination of the constituents of the tested formulation by *A. anguilla*, namely deltamethrin, in contrast to what has been stated for 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.

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Conflict of interests

The authors declare that there are no conflicts of interest.

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Chapter 3

Progression of DNA damage induced by a glyphosate-based herbicide in fish (*Anguilla anguilla*) upon exposure and post-exposure periods – insights into the mechanisms of genotoxicity and DNA repair

3. Progression of DNA damage induced by a glyphosate-based herbicide in fish (*Anguilla anguilla*) upon exposure and post-exposure periods – insights into the mechanisms of genotoxicity and DNA repair

Abstract

Roundup® is a glyphosate-based herbicide widely used with both agricultural and non-agricultural purposes, which has been demonstrated to represent a risk to non-target aquatic organisms, namely fish. Among the described effects to fish, genotoxicity has been pointed out as one of the most hazardous. However, the genotoxic mechanisms of Roundup® are not entirely understood as well as the involvement of the oxidative DNA damage repair system. Hence, this work aimed to improve the knowledge on the progression of DNA damage upon short-term exposure (3 days) and post-exposure (1 - 14 days) periods in association with DNA repair processes in *Anguilla anguilla* exposed to Roundup® (58 and 116 $\mu g L^{-1}$). DNA damage in hepatic cells was evaluated by the comet assay improved with the DNA-lesion specific endonucleases FPG and EndoIII. In order to evaluate the oxidative DNA damage repair ability, an *in vitro* base excision repair (BER) assay was performed, testing hepatic subcellular extracts. Besides the confirmation of the genotoxic potential of this herbicide, oxidative damage was implicit as an important mechanism of genetic damage, which showed to be transient, since DNA integrity returned to the control levels on the first day after cessation of exposure. An increased capacity to repair oxidative DNA damage emerging in the post-exposure period revealed to be a crucial pathway for the *A. anguilla* recovery; nevertheless, DNA repair machinery showed to be susceptible to inhibitory actions during the exposure period, disclosing another facet of the risk associated to the tested agrochemical.

Keywords

Roundup®; comet assay; genotoxicity; DNA damage recovery; oxidative DNA damage repair; BER assay

3.1 Introduction

Glyphosate-based products are among the most used herbicides worldwide due to its efficacy and cost effectiveness. These products are post-emergent, systemic and non-selective herbicides, adopted for both agricultural and non-agricultural purposes (Monheit 2007). Roundup® appears as one of the most popular commercial formulations in which glyphosate is included as N-(phosphonomethyl) glycine (active ingredient), also containing polyethoxylene amine as surfactant. These agrochemicals easily reach the water bodies due to surface runoff and soil leaching or spray-drift during application. Consequently, non-target organisms, namely fish, might be affected. Among the different deleterious effects detected in fish following exposure to glyphosate (or glyphosate-containing products) (Fan et al. 2013; Harayashiki et al. 2013; Langiano and Martinez 2008; Modesto and Martinez 2010), genotoxicity has been pointed out by different authors as one of the most hazardous (Cavalcante et al. 2008; Çavas and Könen 2007; Ghisi and Cestari 2013; Grisolia 2002; Guilherme et al. 2012a).

DNA integrity can be affected by a combination of factors, including spontaneous cell processes such as reactive oxygen species (ROS) production and exposure to environmental genotoxicants (Geacintov and Broyde 2010). The ultimate effect of the latter agents strongly depends on the exposure length and concentration, as well as on the associated variations on the balance between pro-genotoxic processes (e.g. bioactivation, ROS over-generation) and anti-genotoxic actions such as detoxification/elimination of the agent/metabolites, antioxidant defenses and DNA repair (Ioannides and Lewis 2004). In fact, DNA repair system is a key factor on preventing severe genetic damage (e.g. mutations, DNA strand breaks and chromosomal aberrations). Thus, the exposure to environmental genotoxicants does not necessarily result in DNA damage if the repair system is not compromised (Shugart and Theodorakis 1996). DNA repair can be divided into two main mechanisms: direct reversal of DNA damage or removal of the damaged section (followed by re-synthesis of the excised region) (Weaver 2008). By the latter process, usually called excision repair, the damaged DNA is recognized and removed either as free bases or as nucleotides. Nucleotide excision repair (NER) generally deals with severe changes to bases and removes a large section of DNA (oligonucleotide) containing those damaged bases (Weaver 2008). Base excision repair (BER) is more prevalent, working on subtle damage in DNA bases, being the most important mechanism repairing base alterations, including deaminated or oxidized bases. Basically, specific DNA glycosylases recognize damaged bases, removing them and leaving apurinic/apyrimidinic (AP) sites, where AP endonucleases will

cut the phosphodiester backbone. Then, DNA polymerase will fill in with new DNA followed by the sealing of the remaining nick by DNA ligase (Kienzler et al. 2013b; Weaver 2008). According to Collins (2001), the single cell gel electrophoresis (comet assay) can be adopted, not only to signal DNA strand breaks, but also to evaluate the DNA repair ability of a given tissue through the *in vitro* assay for BER. Briefly, a DNA substrate containing specific lesions is incubated with a cell-free extract prepared from the tissue under analysis. The accumulation of breaks due to the incubation with extract is a measure of DNA repair activity in the tissue (Azqueta et al. 2013). This *in vitro* BER assay has been described as an useful tool to assess the effects of environmental contaminants on the DNA damage repair capability of exposed organisms (Kienzler et al. 2013a).

Despite the growing number of publications identifying the Roundup® genotoxic potential to fish (Cavalcante et al. 2008; Çavas and Könen 2007; Ghisi and Cestari 2013; Grisolia 2002; Guilherme et al. 2012a), the underlying mechanisms are not entirely understood. There are a few studies stating that Roundup® is able to induce oxidative stress in liver cells of fish (Langiano and Martinez 2008; Lushchak et al. 2009). Recently, Guilherme et al. (2012a, b) found evidences of DNA oxidative damage (as oxidized purines) in blood and liver cells of *Anguilla anguilla* exposed to Roundup®, although no changes were noticed on the antioxidant system of the latter tissue. Oxidative stress has been presented as an important source of DNA damage by pesticides, but also as a cause of impairments in the DNA repair proteins system (Muniz et al. 2008). However, the modulation of DNA repair activity by water contaminants in fish is a topic still scarcely explored. The only examples concern the application of BER assay in fish cell lines after exposures to 3-aminobenzamide, cadmium and tributyltin (Kienzler et al. 2013b) and the detection of tissue-specificities of BER activity in *Xiphophorus* species (Walter 2001). To the authors' knowledge, no study addressed DNA repair function in fish exposed to Roundup®. Therefore, this subject can be regarded as a significant lacuna on the understanding of its genotoxic mechanisms and a limitation on the capacity to predict the consequences of exposure to this herbicide, justifying redirect of the research focus in that direction.

Taking as a departing point the genotoxicity of Roundup® already demonstrated in fish (Cavalcante et al. 2008; Çavas and Könen 2007; Ghisi and Cestari 2013; Grisolia 2002; Guilherme et al. 2010), as well as the involvement of oxidative stress (Guilherme et al. 2012a, b), the major purpose of this work was to improve the knowledge on the progression of DNA damage upon exposure and post-exposure periods in association with processes of oxidative DNA damage repair. Hence, *Anguilla anguilla* was exposed for

3 days to Roundup® (58 and 116 μgL^{-1}) and allowed to recover in herbicide-free water for 1, 7 and 14 days. DNA damage in liver cells was evaluated by the comet assay improved with the nucleoid digestion with endonucleases FPG (formamidopyrimidine DNA glycosylase) and EndoIII (endonuclease III) to detect oxidized purines and pyrimidines, respectively. In order to estimate the capacity of oxidative DNA damage repair, an *in vitro* BER assay was carried out with liver cells extracts, using substrate cells treated with paraquat (a standard inducer of oxidative damage).

3.2 Materials and Methods

3.2.1 Chemicals

The commercial formulation of glyphosate [N-(phosphonomethyl) glycine] used - Roundup® - is distributed by Bayer CropScience Portugal, containing isopropylammonium salt of glyphosate at 485 gL^{-1} as the active ingredient (equivalent to 360 gL^{-1} or 30.8% of glyphosate) and polyethoxylene amine (16%) as surfactant. DNA lesion-specific repair enzymes, namely FPG and EndoIII, were purchased from Professor Andrew Collins (University of Oslo, Norway). All the other chemicals were obtained from the Sigma-Aldrich Chemical Company (Spain).

3.2.2 Test animals and experimental design

A. anguilla specimens with an average length of 25 ± 3 cm and weight of 32 ± 5 g (yellow eel stage) were captured from an unpolluted area of Aveiro Lagoon - Murtosa, Portugal. Eels were acclimated to the laboratory for 15 days, maintained in 60-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 7.5 ± 0.1, nitrate 21 ± 0.6 mgL^{-1}, nitrite 0.04 ± 0.01 mgL^{-1}, ammonia 0.2 ± 0.05 mgL^{-1}, dissolved oxygen 8.2 ± 0.6 mgL^{-1}. During this period, fish were daily fed with fish roe.

The experiment was carried out in 60-L aquaria, in a semi-static mode, under the same conditions described for the acclimation period. After this period, 96 specimens were divided into 12 experimental groups, corresponding to three test conditions and four exposure/post-exposure times. In the exposure period, two groups of eels were exposed to 58 (R1) and 116 μgL^{-1} (R2) of Roundup® (corresponding to 18 and 36 μgL^{-1} of glyphosate, respectively) during 3 days. Thereafter, fish were transferred to herbicide-free water and sampled at 1, 7 and 14 days (post-exposure period). In parallel, groups of fish were kept in clean water and sampled at the same times (control groups). Each experimental group was comprised by 8 fish (8 fish per condition/time). Water medium in

both exposure and post-exposure periods was daily renewed (100%). A stock solution of Roundup® was prepared (in deionised water) just before addition to the aquaria.

Fish were not fed during the exposure period. In the post-exposure period, fish were daily fed with fish roe, except at the day before samplings that were not fed. At each sampling time, fish were sacrificed by cervical transaction and bled. Liver was collected and washed in ice-cold phosphate-buffered saline (PBS). A small portion of liver was immediately processed for comet assay and the remainder tissue was frozen in liquid nitrogen and kept at -80 ºC until further procedures for the comet assay adjusted to assess BER activity.

3.2.3 Comet assay

The cell suspension of the liver was obtained by mincing briefly a portion of the tissue with a pair of fine scissors in 1mL of PBS (Hartmann et al. 2003). The alkaline version of the comet assay was performed according to Collins (2004) methodology with slight modifications, as adapted by Guilherme et al. (2012a), as well as the proper adjustments to the extra step, concerning the nucleoids digestion with endonucleases. A system with eight gels per slide was adopted, based on a model of twelve gels created by Shaposhnikov et al. (2010), in order to improve the assay output. Therefore, 20 µL of liver cell suspension containing approximately $2x10^4$ cells were mixed with 70 µL of 1% low melting point agarose in PBS. Eight drops of 6 µL were placed onto the glass microscope slide, precoated with 1% normal melting point agarose, as two rows of 4 (4 groups of 2 replicates), without coverslips, each drop/gel containing approximately 1,500 cells. The slides were left ±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, and kept overnight. 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 4ºC.

In total, 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). The third set (3) was incubated only with enzyme buffer. Hence, 30 µL of each enzyme diluted in enzyme buffer (and only buffer in the third set) were applied to each mini-gel (i.e. 120 µL per each group of 4 mini-gels) with coverslip, and the slides were incubated at 37 ºC during 30 min in a humidified chamber. Then, slides were immediately placed in the electrophoresis tank, immersed in electrophoresis solution for ±20 min (alkaline treatment). Electrophoresis was

performed during 15 min at a fixed voltage of 25 V, a current of 300 mA, which results in 0.7 Vcm^{-1} (achieved by adjusting the buffer volume in the electrophoresis tank). The slides were stained with ethidium bromide (20 μgmL^{-1}).

3.2.4 BER assay

This *in vitro* DNA repair assay consisted in a comet assay applied to substrate cells previously treated with an oxidative lesion inducing agent and submitted to a cell-free extract of liver. This extract performs the initial step of DNA repair, i.e. the incision on the DNA molecule of substrate cells presenting specifically oxidative lesions, generating breaks. The protocol adopted was adjusted from the methodology described by Collins (2001) and Gaivão et al. (2009). Briefly, blood freshly collected from unexposed eels was used to provide substrate cells. The system of eight gels per slide was adopted with the above described methodology. After lysis, nucleoids were treated with paraquat (PQ; 0.05 µM) that was used as a standard inducer of oxidative DNA damage (Ali et al. 1996). Thus, 30 µL of PQ diluted in PBS were applied to each mini-gel (i.e. 120 µL per each group of 4 mini-gels) with coverslip, and left at 4 ºC for 15 min. Then, PQ-treated slides were washed 2 times with enzyme buffer and divided into 2 sets: (1) one submitted to the cell-free extract, and (2) other submitted to a control solution (extraction buffer and Triton X-100).

In order to confirm the induction of oxidative DNA damage by PQ, one slide (with 8 mini-gels) was treated with PQ (as previously described) and afterwards treated with FPG. Another slide was left out of the PQ treatment and not submitted to any enzymatic reaction, in order to provide the DNA damage baseline of the substrate cells.

To prepare liver extracts, a tissue portion (approximately 50-100 mg) was thawed and resuspended in extraction buffer [45 mM 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES), 0.4 M KCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM dithiothreitol, 10% glycerol, adjusted to pH 7.8 using 6 M KOH] diluted 3x in H_2O. Then, 500 µL of the resulting cell suspension were collected, snap-frozen in liquid nitrogen, thawed again, and mixed with 150 µL of a solution of triton X-100 in extraction buffer (1:100). Thereafter, this solution was centrifuged at 11,200 g for 5 min at 4 ºC, to remove nuclei and cell debris, and the supernatant was collected. Total protein concentration was determined according to the Biuret method (Gornall et al. 1949) and the final extract was obtained after the appropriate dilution to get 0.3 mgL^{-1} of protein.

To allow the activity of repair enzymes, 20 µL of cell-free extract were applied to each mini-gel (i.e. 80 µL per each group of 4 mini-gels) with coverslip, and the slides were incubated at 25 ºC (adjusted to fish body temperature) in a humidified chamber, during 30

min. At the same time, the slide treated with FPG was incubated at 37ºC. Then, slides were immediately placed in the electrophoresis solution for 20 min (alkaline treatment) and the electrophoresis and subsequent steps occurred as previously described (see point 3.2.3).

3.2.5 Slide analysis

It was evaluated the genetic damage index (GDI) in slides treated with FPG or EndoIII, corresponding to the parameters GDI_{FPG} and $GDI_{EndoIII}$, respectively. Additionally, in order to assess the DNA breaks corresponding specifically to net enzyme-sensitive sites (NSS), slides not submitted to endonuclease treatment were also scored. These scores were subtracted to GDI_{FPG} or $GDI_{EndoIII}$ values resulting in the parameters expressed as NSS_{FPG} and $NSS_{EndoIII}$, respectively.

Fifty nucleoids were observed per each gel, using an Olympus BX 41 fluorescence microscope (400x of magnification). The nucleoids were classified by visual scoring into 5 comet classes, according to the tail length and intensity from 0 (no tail) to 4 (almost all DNA in tail) (Collins 2004). The final score – expressed as "arbitrary units" in a range of 0-400 – was obtained by multiplying the mean percentage of nucleoids in each class by the corresponding factor, according to this formula:

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

In what concerns to BER assay, slides were scored using the same procedures described above. BER overall values correspond to DNA breaks resulting from the additive action of PQ treatment and cell-free extract. At this analysis, the two previously described sets of slides were scored: (1) slides submitted to the cell-free extract, to quantify the extract-induced breaks after PQ treatment; (2) slides submitted to a control solution to establish the level of breaks in the substrate cells exposed to PQ (PQ-induced damage). One mini-gel submitted to PQ treatment (and control solution) was scored per each mini-gel submitted to PQ treatment and extract incubation. Thus, as BER results it was shown the overall breaks (given by the direct scoring of slide 1), the PQ-induced breaks (given by the direct scoring of slide 2), and net extract-induced breaks (NEB) obtained by the subtraction of the two previous values.

3.2.6 Statistical analysis

Statistica 8.0 software was used for statistical analysis. All data were first tested for normality and homogeneity of variance to meet statistical demands. For each sampling moment, one-way Analysis of Variance (ANOVA) was applied, followed by a post hoc Tukey test, to compare all the test groups between them (within the same exposure/post-exposure time). The differences between means were considered significant when $p < 0.05$ (Zar 1996).

3.4 Results

3.4.1 Comet assay

These results concern the comet assay improved with the extra-step involving DNA lesion-specific endonucleases (FPG and EndoIII). Taking into account the exposure period, the levels of overall damage were significantly higher in the exposed groups (R1 and R2), comparing to the control, for both GDI_{FPG} and $GDI_{EndoIII}$ parameters (Figs. 1A and 2A). In addition, $GDI_{EndoIII}$ levels were significantly higher in R1 group in relation to R2. Considering specifically the DNA breaks corresponding to net endonuclease-sensitive sites following 3-day exposure (Figs. 1B and 2B), liver cells from fish exposed to the lowest concentration of Roundup® (R1) displayed significantly lower NSS_{FPG} levels in comparison to the control, while $NSS_{EndoIII}$ revealed significantly lower levels for both R1 and R2 groups.

Considering the post-exposure period, GDI_{FPG} and $GDI_{EndoIII}$ levels in exposed groups were no longer different from the respective control (Figs. 1A and 2A), with the exception of GDI_{FPG} in R2 group after 1 day in herbicide-free water, which showed lower DNA damage values relatively to the control and R1 groups. In what concerns to net endonuclease-sensitive sites (Figs. 1B and 2B), in the first day post-exposure, NSS_{FPG} revealed significantly lower levels in R2 group comparing to both control and R1 groups, whereas for $NSS_{EndoIII}$, the same fish group (R2) showed a decrease in relation to the other exposed group (R1). After 7 and 14 days post-exposure, significant alterations in NSS_{FPG} and $NSS_{EndoIII}$ parameters were no longer observed.

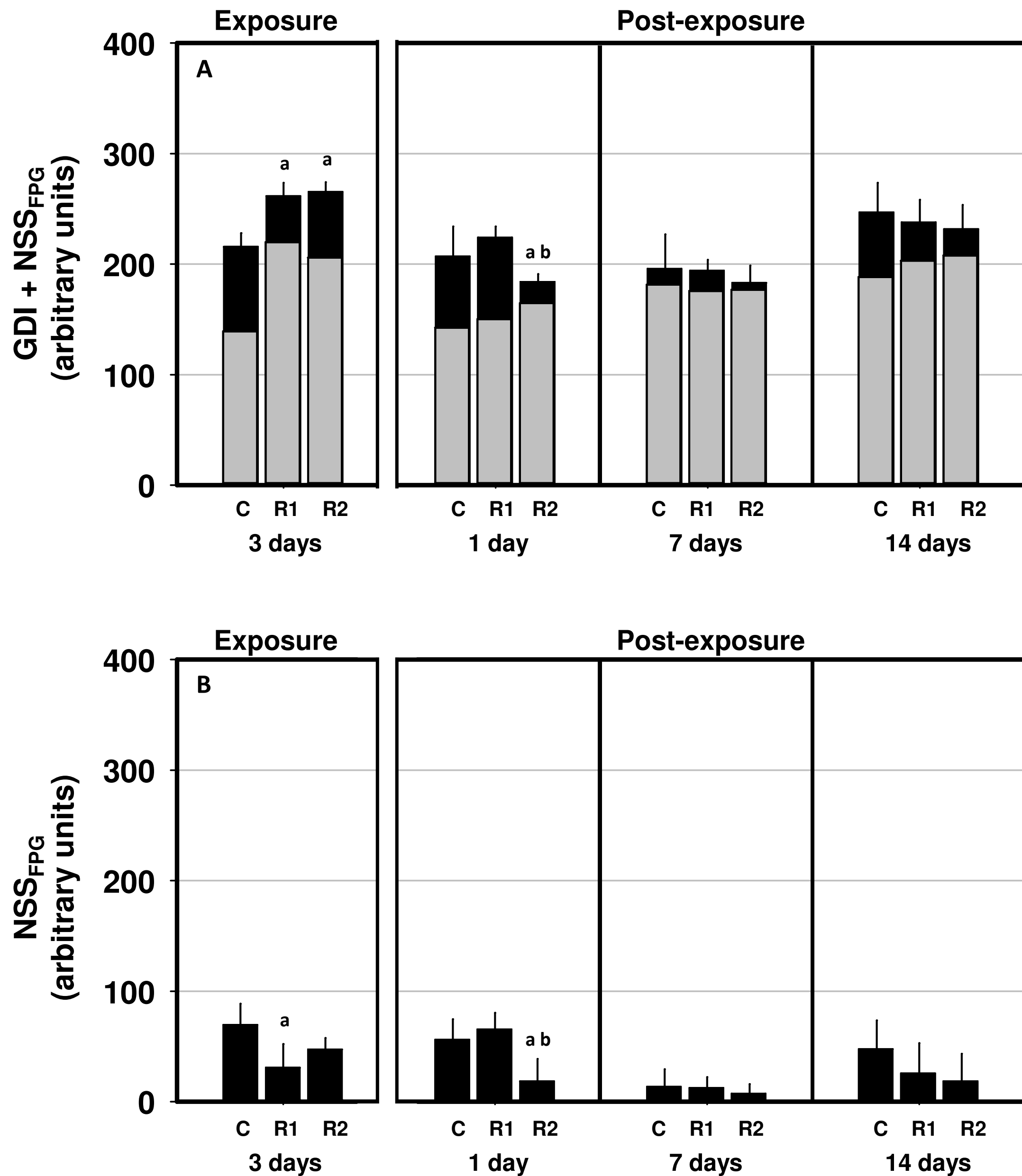

Figure 1 – Mean values of DNA damage, measured by the comet assay in liver cells of *A. anguilla* exposed to 58 and 116 μgL^{-1} of Roundup® (R1, R2, respectively) during 3 days (Exposure) and 1, 7 and 14 days after transference to herbicide-free water (Post-exposure). Values resulted from the assay with an extra step of digestion with formamidopyrimidine DNA glycosylase (FPG) to detect oxidized purine bases: **(A)** overall damage (GDI_{FPG}) and partial scores, i.e. genetic damage indicator (GDI) after the standard comet assay (grey) and additional DNA breaks corresponding to net FPG-sensitive sites (NSS_{FPG}; black); **(B)** NSS_{FPG} alone. Statistically significant differences ($p<0.05$) are: (a) *versus* control and (b) *versus* R1 (within the same exposure/post-exposure time). Bars represent the standard deviation.

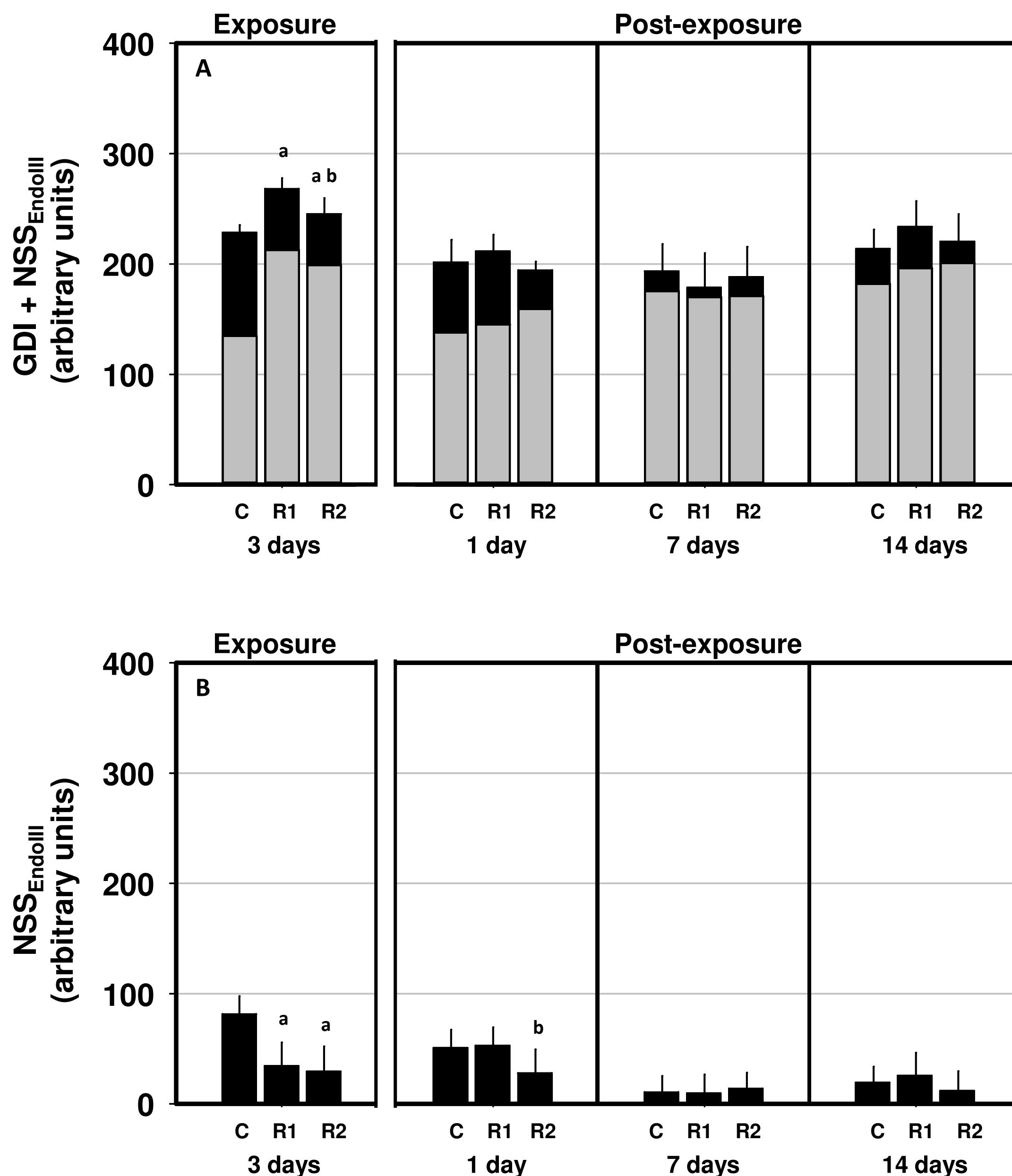

Figure 2 – Mean values of DNA damage, measured by the comet assay in liver cells of *A. anguilla* exposed to 58 and 116 $\mu g L^{-1}$ Roundup® (R1, R2, respectively) during 3 days (Exposure) and 1, 7 and 14 days after transference to herbicide-free water (Post-exposure). Values resulted from the assay with an extra step of digestion with endonuclease III (EndoIII) to detect oxidized pyrimidine bases: **(A)** overall damage ($GDI_{EndoIII}$) and partial scores, i.e. genetic damage indicator (GDI) after the standard comet assay (grey) and additional DNA breaks corresponding to net EndoIII-sensitive sites ($NSS_{EndoIII}$; black); **(B)** $NSS_{EndoIII}$ alone. Statistically significant differences ($p<0.05$) are: (a) *versus* control and (b) *versus* R1 (within the same exposure/post-exposure time). Bars represent the standard deviation.

3.4.2 BER assay

The DNA breaks increment in substrate cells resulting from BER activity in liver extracts is depicted in figure 3, emphasizing the NEB (the level of breaks after PQ treatment is shown by the dashed line).

It was also scored the baseline of DNA breaks in substrate cells (without PQ treatment or enzymatic incubation), showing an average value of 162.38 ± 20.87. In order to demonstrate the induction of oxidative DNA damage by PQ, it was performed an incubation of PQ-treated slides with FPG (since it provokes breaks at oxidized bases), showing an average level of 267.25 ± 28.13.

The analysis of figure 3 shows that liver extracts from eels exposed to both concentrations of the herbicide during 3 days induced significantly less DNA breaks in comparison to control. However, when it was observed the hepatic BER activity of fish after 1 day in herbicide-free water, the pattern was the opposite, i.e. R1 and R2 groups revealed an increase of the DNA breaks, relatively to the control group. At 7 and 14 days post-exposure, no significant alterations were found.

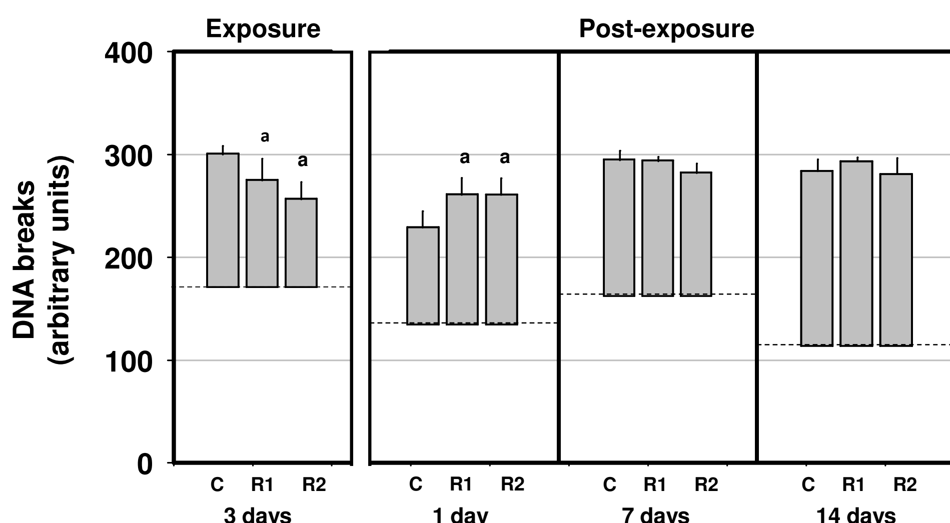

Figure 3 – Mean values of DNA breaks, measured by the BER assay in PQ-treated blood cells (substrate cells) of *A. anguilla*, resulting from the activity of liver extracts of eels exposed to 58 and 116 µgL^{-1} Roundup® (R1, R2, respectively) during 3 days (Exposure) and 1, 7 and 14 days after transference to herbicide-free water (Post-exposure). Grey columns represent net extract-induced breaks (NEB) fitted on the level of PQ-induced breaks (dashed line). The upper level of the columns represents the overall level of breaks (PQ-induced plus NEB). Statistically significant differences ($p<0.05$) for NEB are: (a) *versus* control (within the same exposure/post-exposure time). Bars represent the standard deviation for NEB.

3.5 Discussion

The detection of Roundup® (as glyphosate acids equivalent) at surface waters in a range between 0.01 and 0.7 mgL^{-1} (Peruzzo et al. 2008), reaching 1.7 mgL^{-1} after a direct application in aquatic systems (Horner 1990), raised a wide concern about the possible effects of this herbicide in non-target aquatic organisms. Its interference with DNA integrity has been presented as one of the most critical effects (Cavalcante et al. 2008; Çavas and Könen 2007; Guilherme et al. 2012a), considering the potential impact on the organisms´ health and survival. However, the assessment of the actual magnitude of risk posed by these agrochemicals to genomic integrity of fish, as well as the determination of a subsequent ecological impact, require a deeper knowledge on the underlying mechanisms of damage and on the ability to repair the DNA damage induced.

Owing to the crops seasonality and subsequent periodic/seasonal application of herbicides, their input to the aquatic systems is typically intermittent. This aspect, combined with fish mobility and avoidance behaviour, makes probable that fish exposure to this type of contaminants can be short (time-scale of days) and followed by a period of permanence in non-contaminated areas. Bearing this in mind, it emerges a need to assess genotoxic endpoints in fish after removal of the contamination source (Bony et al. 2008), addressing in particular the response of DNA repair system. Nevertheless, this perspective has been scarcely explored with fish and, thus, the present study appears as the first work evaluating the progression of DNA damage and modulation of DNA repair activity in cells of fish following exposure to Roundup® as well as in the corresponding post-exposure period.

3.5.1 DNA damage assessed through comet assay

The Roundup® genotoxic potential to fish has been described by several authors (Cavalcante et al. 2008; Çavas and Könen 2007; Ghisi and Cestari 2013; Grisolia 2002; Guilherme et al. 2012a). In accordance to those studies, in the current work Roundup® showed to induce DNA damage (measured both as GDI_{FPG} and $GDI_{EndoIII}$) in hepatic cells of *A. anguilla* exposed to two environmentally realistic concentrations (R1 – 58 and R2 – 116 μgL^{-1}) during 3 days. However, when DNA strand breaks resulting specifically from FPG (NSS_{FPG}) or EndoIII ($NSS_{EndoIII}$) activities are under analysis, lower levels were observed in treated fish. This can be regarded as a surprising result and, in a first analysis, it can be unadvisedly interpreted as reflecting the absence of an oxidative challenge to exposed fish. However, this interpretation would fit better in a response profile where no differences are detected between exposed and unexposed organisms.

Therefore, in the current case, it should be suggested that exposed fish evolved defensive and compensatory processes to cope with the oxidative pressure posed by Roundup®, whose efficacy was able to bring down the DNA oxidative damage below the control levels. Though this is not a classical pattern of response in toxicology, it has been often reported in the literature. For instance, lower levels of lipid peroxidation were observed in Roundup®-treated fish in comparison with the control group, either in brain (Glusczak et al. 2007) or in liver (Harayashiki et al. 2013). In both cases the results were explained by a stimulation of the antioxidant scavenging capacity. In this direction, it is plausible that under a low/moderate attack by xenobiotics, fish can trigger protective mechanisms able to prevent the DNA damaging effect of an external threat as well as of that caused by endogenous factors. Hence, two mechanisms can be hypothesized (alternatively or in combination): after the herbicide uptake, cells responded by (i) enhancing its DNA repair capacity and/or (ii) mobilizing the antioxidant system as a response to ROS over-generation, reducing the cell vulnerability towards oxidative damage induced by the agrochemical. The hypothesis involving DNA repair stimulation was not supported by the present BER results, as it will be discussed below (see point 3.5.2), thereby strengthening the second hypothesis.

After transference to herbicide-free water, fish were able to reverse the genetic damage, evaluated as GDI_{FPG} and $GDI_{EndoIII}$, up to the control levels (on the first day post-exposure and thereafter). This recovery should be regarded as a multifactorial process, being firstly determined by the (i) toxicokinetics of the genotoxicants, and then by variables like (ii) the cell turnover or apoptosis in the tissue, and (iii) the evolution of antigenotoxic processes such as DNA repair and antioxidant actions.

In an attempt to clarify if the suppression of a direct genotoxic pressure resulting from chemical elimination may have contributed to a damage decline, it should be considered that the degradation of glyphosate, the active ingredient of Roundup®, is relatively rapid, with a half-life in rat plasma in a range of 9.99 - 14.38 hours (Anadón et al. 2009), thus displaying a low bioaccumulation potential (WHO 1994). Therefore, a substantial reduction of glyphosate (or its metabolites) in the liver as a determinant condition for damage recovery was probably achieved from the first day post-exposure onwards.

It has been stated that hepatic cells present a slow turnover rate - approximately 1 year or more (Alberts et al. 2008). Therefore, the removal of cells presenting genetic damage cannot be hypothesized to explain the DNA integrity recovery observed in a time-scale of 1 - 14 days. Therefore, a superimposition of antigenotoxic processes in relation to

progenotoxic pressures should be presented as the main explanation to the recovery achieved by the eels after cessation of exposure. In this direction, the current BER data confirmed the involvement of an incremented DNA repair activity (see point 3.5.2) on the first day post-exposure. Though the antioxidant capacity was not evaluated in the present study, its contribution should also be considered, mainly in R2 group (1 day post-exposure) where the damage levels (especially as GDI_{FPG}) decreased to values below the control group. Since R1 and R2 groups displayed similar DNA repair capacity, in the case of R2 group the additional factor that can explain the decreased DNA damage is probably related to antioxidants mobilization. Additionally, an overall analysis of the present results suggests the existence of different threshold limits for the modulation (induction or inhibition) of the different defensive lines, namely DNA repair and antioxidant systems.

Evaluating specifically the oxidative damage translated as NSS_{FPG} and $NSS_{EndoIII}$, after 1 day in herbicide-free water, hepatic cells of eels previously exposed to the highest concentration (R2) still showed levels of damage lower than the control. Once more, this effect may result from the activated cell defence mechanisms of (i) DNA repair or (ii) antioxidant system.

After 7 and 14 days of fish permanence in herbicide-free water, DNA damage was kept at the control level, confirming the cell ability to deal with the damage previously induced through the integration of the different defence mechanisms. However, this apparent recovery should be carefully interpreted since DNA strand breaks might not be the only DNA modification induced by the agrochemical. Even if this type of damage can be repaired, DNA mutations can remain in the cells.

3.5.2 DNA repair assessed by BER assay

Analyzing the cell behaviour in what concerns to BER assay, after 3 days exposure to Roundup®, the occurrence of lower levels of DNA breaks in substrate cells incubated with extracts from exposed fish (when compared to control) indicated that the oxidative DNA damage repair ability of liver decreased. Two hypotheses can be formulated to explain this result: an inhibition of DNA repair enzymes (as a toxic action associated to Roundup® exposure) or a sub-expression of this enzymatic system due to a lack of pro-oxidant pressure and subsequent lower oxidative DNA damage.

In what concerns the BER responses during the post-exposure period, particularly after 1 day in herbicide-free water, it was clear an increased capacity of exposed fish (both R1 and R2) to repair oxidative DNA damage. This is a demonstration of fish adaptive skills, enabling it to prevent neo-generated damage in this period as well as to repair damage

induced in the precedent exposure period. On the other hand, these results are also an evidence of a pro-oxidant status induced by Roundup® and its potential to oxidatively damage DNA.

It is not expectable that the oxidative pressure on the first day post-exposure was higher than after three consecutive days of exposure to the agrochemical. Moreover, an increased repair of oxidative DNA damage was observed (at 1-day post-exposure) without a concomitant expression of oxidative DNA damage (as NSS_{FPG} and $NSS_{EndoIII}$). Thus, and bringing to the fore again the reduction of repair activity observed after 3 days exposure, the hypothesis of an enzymatic inhibition gains consistency. Consequently, the activation of the antioxidant system as a response to ROS increase seems to be the only defence shield of the cells during exposure period, signalling an increased risk to fish.

The mechanisms of modulation of DNA repair system by pesticides are poorly understood. However, it is known that other contaminants like metals can interfere with BER activity, alongside with an oxidative damage induction (Hartwig et al. 2003). In addition, Au et al. (2010) stated that the exposure to DNA-damaging agents can also affect the components of the vast machinery of DNA repair. Moreover, this study also suggested that both damage at DNA and repair machinery contribute to an increase of cancer risk, enlightening that damage at DNA repair machinery is as deleterious as DNA damage.

At day 1 post-exposure, the inhibitory action was dissipated, probably due to an improvement of the biochemical environment in the liver cells (consequence of a reduction on the levels of genotoxic agents *viz*. Roundup® constituents/metabolites and/or ROS), releasing DNA repair machinery and allowing the increment of activity.

After 7 and 14 days of post-exposure period, no more significant alterations were noticed in the DNA repair capacity of hepatic cells, pointing out a consolidation of the recovery process.

3.6 Conclusions

First of all, the present results confirmed the genotoxic potential of Roundup® to fish, following short-term exposure to environmentally realistic concentrations. The involvement of oxidative processes on the DNA damage detected, though not explicit in the NSS_{FPG} and $NSS_{EndoIII}$ data, was implicit when all the parameters were put in parallel.

In an attempt to shed light on the progression of DNA damage upon exposure and post-exposure periods and association with DNA repair processes, it was demonstrated that:

- The genetic damage measured by the comet assay as GDI_{FPG} and $GDI_{EndoIII}$ on the exposure period showed to be a transient manifestation, since DNA integrity returned to the control level on the first day after cessation of the exposure;

- An increased capacity to repair oxidative DNA damage emerging in the post-exposure period revealed to be a crucial pathway for the *A. anguilla* recovery from the genetic damage induced by this glyphosate-based herbicide; nevertheless, DNA repair enzymes seem to be susceptible to inhibitory actions associated to higher levels of Roundup® constituents/metabolites and/or ROS likely to occur in liver during the exposure period;

- The elucidation of temporal patterns for DNA damage and repair (measured through comet assay) in exposure and post-exposure periods is a complex task, due to a dependence on a diversity of factors following different variation profiles as a function of time and extent of pro-genotoxic conditions.

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.

Conflict of interests

The authors declare that there are no conflicts of interest.

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Chapter 4

Final remarks

4. Final remarks

This chapter aims to integrate, analyze and consolidate the final reflections relatively to the chapters 2 and 3, to meet the main objectives of the present thesis, enunciated at Chapter 1.

Thus, the main final aspects to be emphasized are:

- ✓ The formulations Decis® and Roundup® revealed their genotoxic ability, at environmentally realistic concentrations, since they induced cytogenetic and DNA damage, respectively, to fish *A. anguilla*. In both cases, the damage showed to be of transient nature considering that Decis®-induced cytogenetic damage returned to control levels after 7 days in insecticide-free water, while Roundup®-induced DNA damage was reverted after 1 day in herbicide-free water.

- ✓ In what concerns to Decis® genotoxicity evolution, the reversal of the chromosomal damage was due to the removal of the contaminant and the involvement of a preferential elimination of the existent damaged cells, since the hematological dynamics suffered no alterations, as depicted in the stable immature erythrocytes (IE) frequency.

- ✓ The involvement of oxidative damage in Roundup® genotoxicity was demonstrated, although not directly reflected in an increase of breaks at FPG- and EndoIII-sensitive sites, which would report the oxidized purines and pyrimidines bases, respectively. However, the opposite pattern was observed, with NSS_{FPG} and $NSS_{EndoIII}$ reporting lower levels than control, suggesting that treated fish developed defensive and compensatory processes to cope with the oxidative pressure induced by Roundup®.

- ✓ On the first day of the cessation of the exposure to Roundup®, fish were able to completely reverse the levels of DNA damage due to the increased activity of the DNA repair system, although it seemed to be inhibited during the exposure period.

- ✓ The erythrocytic nuclear abnormalities (ENA) and comet assays were used in order to assess cytogenetic damage (as chromosomal breakage, chromosome segregation abnormalities and/or dysfunctional mitosis) and DNA damage (as single- and double-

strand breaks and alkali labile sites), respectively. Hence, both techniques revealed to be suitable to detect the described types of damage; however, it became clear that both genotoxic endpoints should be used as complementary tools, since each one measures specific damage types that might be detected at different moments concerning exposure/post-exposure periods (Guilherme 2012).

- ✓ The teleost *Anguilla anguilla*, selected as test organism, was appropriate to assess the genotoxicity of Decis® and Roundup®, responding to environmentally realistic concentrations of both pesticides.

- ✓ In what concerns to the monitoring and management practices of pesticides use, the present study demonstrated that aquatic biota is under substantial threat. Further studies on this subject should be encouraged, contributing to the increased knowledge and the assessment of the environmental risk to fish populations. In addition, it should be investigated the real impact of pesticides, considering the fish ability to rapidly reverse the short-term effects. This might help the establishment of stricter regulatory procedures aiming the reduction of pesticides overuse and/or inadvertent applications as well as the choice of less toxic compounds.

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