Universidade de Aveiro Departamento de Biologia
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Unravelling genotoxic effects of pesticides - *in vitro* and *ex vivo* assays as screening tools

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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 da Doutora Sofia Isabel Antunes Gomes Guilherme, Investigadora em Pós-doutoramento do Departamento de Biologia da Universidade de Aveiro e do Centro de Estudos do Ambiente e do Mar, e coorientação da Doutora Helena Cristina Correia de Oliveira, Investigadora em Pós-doutoramento do Departamento de Biologia da Universidade de Aveiro e do Centro de Estudos do Ambiente e do Mar.

Dedico-te todo o meu trabalho, amor e saudade, A.

"When I'm working on a problem, I never think about beauty. I think only how to solve the problem. But when I have finished, if the solution is not beautiful, I know it is wrong."

Richard Buckminster Fuller

o júri

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palavras-chave Genotoxicidade, pesticidas, *ex vivo*, *in vitro*, lagostim, HepG2

resumo

As acções antropogénicas são uma das principais fontes de contaminantes aquáticos presentes no meio ambiente, muitas vezes comprometendo o ecossistema e, consequentemente, os organismos presentes. Os efeitos destes compostos na biota e nos seres humanos devem ser avaliados, adoptando metodologias de confiança. Os métodos comumente usados, como as abordagens *in vivo*, apresentam várias desvantagens. Além disso, a implementação da política dos 3R (Redução, Refinamento e Substituição) foi considerada uma prioridade, reforçando a necessidade de encontrar métodos alternativos. Tendo isto em conta, este trabalho teve como objectivos (i) validar a abordagem *ex vivo*, como uma alternativa na pesquisa animal, (ii) avaliar o potencial genotóxico de três pesticidas químicos, um insecticida (dimetoato), um fungicida (imazalil) e um herbicida (penoxsulam), e do bioinsecticida Turex® nas células de brânquias de lagostim (*Procambarus clarkii*), usando a abordagem *ex vivo*, e também (iii) determinar a citotoxicidade e genotoxicidade, *in vitro*, do Turex®, na linha celular HepG2. A viabilidade celular das células de brânquias e da linha celular HepG2 foi avaliada às 2, 4 e 8 horas e às 24 e 48 horas, respectivamente. A integridade do ADN foi avaliada usando o ensaio do cometa com a incubação usando enzimas de reparação específicas do DNA, nomeadamente a formamidopirimidina DNA-glicosilase (FPG) e a endonuclease III (EndoIII), para avaliar a oxidação de purinas e pirimidinas, respectivamente.

Relativamente à abordagem *ex vivo*, as células de brânquias de lagostim demonstraram ser adequadas apenas durante 2 horas, quando a viabilidade e a integridade do ADN foram consideradas em conjunto. Tendo em conta esta informação, as células das brânquias foram expostas durante 2 horas a concentrações ambientalmente realistas de inseticida dimetoato (20 µg L^{-1}), do fungicida imazalil (160 µg L^{-1}), e do herbicida penoxsulam (23 µg L^{-1}). Adicionalmente, e relativamente à exposição ao bioinsecticida Turex®, duas abordagens distintas foram consideradas: (1) uma exposição *ex vivo* de células de brânquias do lagostim durante 2 horas a cinco concentrações (25, 50, 100, 200 e 400 µg L^{-1}), onde a genotoxicidade foi avaliada usando o ensaio do cometa, e (2) uma exposição *in vitro* da linha celular HepG2 a outras cinco concentrações (250, 500, 1000, 1500 e 2000 µg L^{-1}), durante 24 e 48 horas, onde a citotoxicidade e a genotoxicidade foram avaliadas, usando o teste MTT e o ensaio do cometa, respectivamente.

O dimetoato, o imazalil e o penoxsulam demostraram ser genotóxicos para as células de brânquias de lagostim, apesar de não induzirem dano oxidativo no ADN. Por outro lado, o Turex® não foi capaz de exercer efeitos genotóxicos nas células de brânquias de lagostim, apesar de apresentar genotoxicidade na linha celular HepG2 (apesar de ser apenas sem activação do insecticida e após 48 h). Além disso, este biopesticida demonstrou ser citotóxico (principalmente quando activado e após 48 h) para a linha celular testada.

Em conclusão, a abordagem *ex vivo* demostrou ser adequada, juntamente com o ensaio do cometa, para exposições de 2 horas, quando aplicada a células de brânquias de lagostim. Deste modo, e considerando esta abordagem, a genotoxicidade dos pesticidas dimetoato, imazalil e penoxsulam foi comprovada. Este estudo demonstrou ainda os possíveis efeitos perigosos do Turex® para a linha celular humana (HepG2), direccionando a atenção para a alegada segurança de biopesticidas baseados em *Bacillus thuringiensis*. Consequentemente, é de todo o interesse que estes grupos de biopesticidas sejam investigados mais profundamente, de forma a determinar os possíveis efeitos em sistemas biológicos.

No geral, os resultados obtidos apresentam-se como uma contribuição relevante para o aprimoramento das estratégias de triagem dos efeitos perniciosos de contaminantes, no sentido de as tornar mais rápidas e eficazes. Este trabalho pretende ainda contribuir para a (re)formulação de procedimentos regulatórios, tanto na aplicação de pesticidas como também para o controlo dos possíveis efeitos negativos dos mesmos, de forma a proteger a saúde ambiental e pública.

keywords Genotoxicity, pesticides, *ex vivo*, *in vitro*, crayfish, HepG2

abstract

Anthropogenic actions are one of the main sources of waterborne contaminants in the environment, often compromising the ecosystem and, consequently, inhabiting organisms. Real effects of these compounds to biota and humans must be assessed, adopting reliable approaches. Commonly used methods, such as *in vivo* approaches, come with several disadvantages. Moreover, the implementation of the 3R's politic (Reduction, Refinement and Replacement) has been considered as a priority, reinforcing the need of finding alternative methods. Bearing this in mind, this study intended (i) to validate the *ex vivo* technique, as an alternative in animal research, (ii) to assess the genotoxicity of three chemically-based pesticides, an insecticide (dimethoate), a fungicide (imazalil) and a herbicide (penoxsulam) and the bioinsecticide Turex® to gill cells of *Procambarus clarkii*, using an *ex vivo* approach, and also (iii) to disclose the cytotoxicity and genotoxicity, *in vitro*, of Turex® to the cell line HepG2. Cell viability of crayfish gills and HepG2 cell line was evaluated for 2, 4 and 8 hours and 24 and 48 hours, respectively. DNA integrity was evaluated using the comet assay, improved with DNA lesion-specific repair enzymes, namely formamidopyrimidine DNA glycosylase (FPG) and endonuclease III (EndoIII), to assess purines and pyrimidines oxidation, respectively.

Concerning the ex vivo approach, crayfish gill cells only showed to be suitable considering exposures of 2 hours, when the viability and the DNA integrity were jointly considered. Accordingly, gill cells were exposed for 2 hours, to environmentally realistic concentrations of the insecticide dimethoate (20 µg L^{-1}), the fungicide imazalil (160 µg L^{-1}), and the herbicide penoxsulam (23 µg L^{-1}). Additionally, and concerning the exposure to the bioinsecticide Turex®, two distinct approaches were considered: (1) an *ex vivo* exposure of crayfish gill cells for 2 hours to five concentrations (25, 50, 100, 200 and 400 µg L^{-1}), where its genotoxicity was evaluated using the comet assay, and (2) an *in vitro* exposure of the HepG2 cell line to other five concentrations (250, 500, 1000, 1500 and 2000 µg L^{-1}) for 24 and 48 hours, after which cytotoxicity and genotoxicity was evaluated using the MTT and the comet assays, respectively.

Dimethoate, imazalil and penoxsulam demonstrated to be genotoxic to crayfish gill cells, despite not inducing oxidative DNA damage. On the other hand, Turex® was not able to exert genotoxic effects in crayfish gill cells, despite presenting genotoxicity to the HepG2 cell line (despite only without activation and after 48 h). Moreover, this biopesticide showed to be cytotoxic (mainly with activation and after 48 h) to the tested cell line.

In conclusion, the *ex vivo* approach, when applied to crayfish gill cells, showed its suitability for exposures of 2 hours, when the comet assay was used. Thus, and considering this approach, the genotoxicity of the pesticides dimethoate, imazalil and penoxsulam was proved. This study also demonstrated the possible dangerous effects of Turex® to a human cell line (HepG2), pointing attention to the alleged safety of a *Bacillus thuringiensis*-based biopesticide. Consequently, this type of biopesticides should be further investigated to determine their possible negative effects on biological systems.

Overall, the obtained results might be assumed as a relevant contribution towards the improvement of strategies for a rapid and effective screening of the pernicious effects of contaminants. This study also intended to contribute to (re)formulate regulatory procedures, both for the application of pesticides, as well as for the control of the possible negative effects, protecting the environmental and public health.

Table of contents

1. Introduction

1.1 Pesticides ubiquity

The anthropogenic dependence on agriculture as a source of food has led to the improvement of techniques and chemicals to increase productivity. This need enhanced the use of pesticides, which are applied to destroy, repel or mitigate plagues. Pesticides are usually classified according to their target organisms, leading to over 15 categories of pesticides (Table I), of which three are the most used and will be focused in this work: herbicides, that control unwanted plants, insecticides, that eliminate insects and the respective eggs and larvae, and fungicides, that affect fungi and their spores (NASDA 2014). All these pesticide categories may have two different origins: chemical or biological. Chemically-based pesticides are synthetic chemicals that generally work by directly killing or inactivating pests while, on the other hand, biopesticides are naturally occurring bioactive organisms or substances, that either directly kill or repel pests, or work indirectly by interfering with their reproduction.

Table I. Classification by target pest of most pesticides (obtained from Davaadulam *et al.* 2014).

Pesticide	Target Pest / Function	Pesticide	Target Pest / Function
Acaricide	Mites, ticks	Growth regulator	Regulates insect and plant growth
Algaecide	Algae	Herbicide	Weeds
Anticoagulant	Rodents	Insecticide	Insects
Attractant	Attracts insects or birds	Miticide	Mites
Avicide	Birds	Molluscicide	Snails, slugs
Bactericide	Bacteria	Nematicide	Nematodes
Defoliant	Plant leaves	Piscicide	Fish
Desiccant	Disrupts water balance in arthropods	Predacide	Vertebrate predators
Fungicide	Fungi	Repellent	Repels vertebrates or arthropods
Silvicide	Woody vegetation	Rodenticide	Rodents

1.2 Chemically-based pesticides

According to the U.S. Environmental Protection Agency (EPA) (2017), during the year 2012 the pesticide usage globally at the producer level was around 2.7 million kilograms, of those 49% were herbicides and plant growth regulators, 18% were insecticides and 14% were fungicides (EPA 2017), pointing the most used pesticides' categories.

Despite obvious benefits, pesticides usage may produce negative consequences, since most of these compounds are considered as toxic (Zidan 2015, Merlin *et al.* 2015). Due to their persistence (Chopra *et al.* 2011) and mobility, these compounds may end up dissolved in waterbodies through soil leaching (Belenguer *et al.* 2014, Masiá *et al.* 2013), drift, or by run-off. Subsequently, water bodies may be contaminated, exposing thus non-target organisms. Several studies have demonstrated the effects of pesticides in the environment, from the ability to affect soil microorganisms (Lew *et al.* 2009) and consequently the quality of agroecosystems (Imfeld and Vuilleumier 2012) as, for example, by inducing endocrine disruption in different animal groups (invertebrates, fish, mammals) (Gooding *et al.* 2003, Brar *et al.* 2010, Oskam *et al.* 2003), to affect biodiversity of fauna and flora (Geiger *et al.* 2010) or several other impacts. Adding to this, these chemicals may end up reaching humans and affecting their health, either through an indirect route, by the application and handling of the products (occupational exposure) or through a direct one, by the consumption of contaminated water and food (Kim *et al.* 2017). Recent estimates reported by Food and Agricultural Organization (2000) show that near three million people are poisoned and two hundred thousand dies from pesticide poisoning use each year. Additionally, cancer and some chronic diseases, from neurological (Parrón *et al.* 2011), respiratory (LeVa *et al.* 2006, Zuskin *et al.* 2008) to reproductive disorders (Farr *et al.* 2004, Swan and Sharpe 2006) have been related to pesticide usage.

The three main classes of chemically-based pesticides, according to their use worldwide, are insecticides, fungicides and herbicides.

1.2.1 The insecticide dimethoate

Dimethoate (O,O-dimethyl S-[2-(methylamino)-2-oxoethyl] dithiophosphate) is an organophosphate insecticide, used worldwide against mites and aphids, by inhibiting acetylcholinesterase (Pope 1999). This pesticide has been shown to induce a hepatotoxic effect on the common carp (Singh 2013) and cause cardiac irregularities in the shore crab (Lundebye *et al.* 1997).

1.2.2 The fungicide imazalil

Imazalil (1-[2-(2,4-dichlorophenyl)-2(2-propenyloxy)ethyl]-1H-imidazole) is a fungicide, used to protect harvested crops after harvesting from rotting, while increasing shelf life (Faniband *et al.* 2015), and it interferes with the fungal wall synthesis, inhibiting it (Zega *et al.* 2009). This pesticide demonstrated the ability to impact the reproductive rate of *P. mammillata* and its ascidian larvae (Pennati *et al.* 2006) and it was suggested to have a neurotoxic effect in zebrafish (Jin *et al.* 2016).

1.2.3 The herbicide penoxsulam

As a herbicide, penoxsulam (3-(2,2-difluoroethoxy)-N-(5,8-dimethoxy[1,2,4]triazolo[1,5-c]pyrimidin-2-yl)-α,α,α-trifluorotoluene-2-sulfonamide) is used for the control of broadleaf weeds, sedge, in cultivation of cereals and particularly of rice (Patetsini *et al.* 2013), by inhibiting cell division and subsequent plant growth (EFSA 2009). It has been observed that penoxsulam induced changes in the ROS levels, DNA damage levels, proteins carbonylation in haemocytes and antioxidant capacity of the haemolymph of *Mytilus galloprovincialis* (Patetsini *et al.* 2013), and it affected the level of different oxidative damage indicators both in brain and muscle tissue of *Rhamdia* sp and *Cyprinus carpio* (Murussi *et al.* 2014).

1.3 Biological pesticides: a better alternative?

In addition to chemically-based pesticides, biological agents can also be used to control plagues. The usage of biological agents as biopesticides is believed to have begun in the 17th century with the use of plant extracts, specifically nicotine, to control plum beetles (Jindal 2017). Biopesticides are based on naturally occurring materials or substances, such as animals (e.g. nematodes), microorganisms (e.g. *Bacillus thuringiensis*), plants (e.g. *Chrysanthemum*), minerals and their products (e.g. phytochemicals and microbial products), because are thought to be a safer choice for the environment than conventional pesticides (Leahy *et al.* 2014, Kumar and Singh 2015, Kumar 2012). Biopesticides may be divided into three major classes, biochemical pesticides, microbial pesticides and plant-incorporated-protectants (PIPs). The first-class accounts for naturally occurring compounds that control pests in a non-toxic process, such as the use of insect sex pheromones that interfere with mating, or scented plant extracts that attract insects to traps (EPA 2016). Microbial pesticides, as the name suggests, have as active substance microorganisms or their compounds, such as bacteria or fungi, and can affect different types of pests according to each active substance (EPA 2016). The most widely used microbial pesticides are the subspecies and strains of *Bacillus thuringiensis* (Bt), that affect one or more related species of insect larvae (Leahy *et al.* 2014, Kumar and Singh 2015). It has been used since the 19th century until today and the first commercial product was available in 1938 in France (Jindal 2017). At last, the PIPs are substances produced by genetically modified plants, where genetic material that provides pesticidal action was incorporated. As an example, the gene for the Bt pesticidal protein can be introduced into the plant's genetic material, providing protection against a specific pest (EPA 2016).

Biopesticides are considered safer than chemically-based ones since they are normally less toxic and more specific since they only affect the target organisms and possibly some closely related ones (EPA 2016). Accordingly, most of them have the requirements to be considered

either as a low-risk active substance, or a basic substance (Czaja *et al.* 2015). According to the Annex VI of Regulation (EC) No. 1095/2007, most biopesticides can be considered to not have harmful effects to humans, animals or any unacceptable effects on the environment (Czaja *et al.* 2015). Adding to this, normally small quantities of biopesticides are required to have an effect, and they often decompose at a fast rate. However, their application should be well thought out and following the pesticide labelling (EPA 2016). Biopesticides can also be part of the Integrated Pest Management (IPM) program, which is an approach to improve pest management using a combination of common-sense practises (EPA 2017, Kumar and Singh 2015). It combines the knowledge about the pest and its interaction with the environment and the available pest control methods, resulting in a combination of techniques that is efficient, cheaper and safer to the environment, to the population and to the crop/plantation (EPA 2017, Kumar 2012). However, biopesticides also have some disadvantages, including a generally smaller and/or slower effectiveness compared to conventional pesticides, and often a reduced shelf life and persistence in the environment (Kumar 2012, Copping and Menn 2000).

Due to the general conception of biopesticides having a very narrow spectrum of action, a scarce number of studies in different organisms have been performed and of those, few conclude that there is a negative effect (Copping and Menn 2000).

Beyond some few examples, there is a lack of studies directed to the biopesticides effects in non-target organisms, including humans, which needs to be fulfilled, being one of the targets proposed for the work developed herein.

1.3.1 What do we know about Turex®?

Turex® is a microbial biopesticides, with insecticide properties, and having *Bacillus thuringiensis* (Bt) subspecies *aizawai* strain GC-91 (Bta GC-91) as the active substance. Bt is an aerobic, gram-positive spore-forming soil bacterium, naturally present in the environment, that produces, during sporulation, insecticidal crystalline inclusions containing proteins designated as protein crystals (cry toxin) (Clark *et al.* 2005, Aronson and Shai 2011). These crystalline inclusions act when the present δ-endotoxins binds to specific receptors on gut epithelial cells of the insect at a basic pH, inserting into the cell membrane. Then, the production of more spores takes place, that will consequently determine cell lysis, leading to the starvation and eventual death of the animal (Clark *et al.* 2005, Van Rie 2000) (Fig. 1).

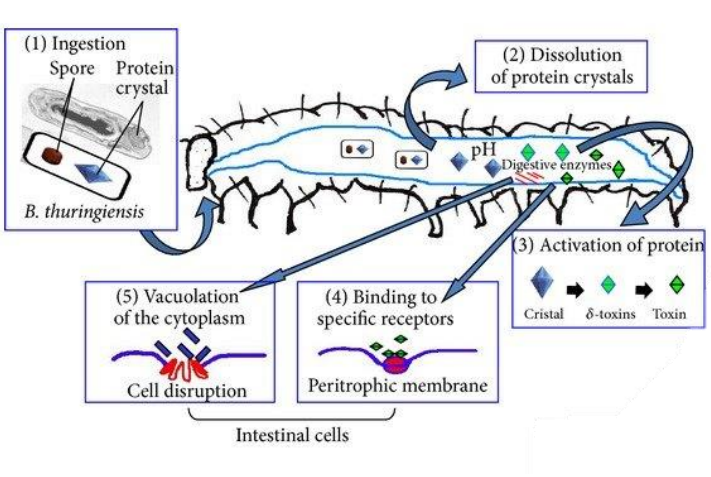

Fig. 1 - Mode of action of *Bacillus thuringiensis*: (1) ingestion of bacteria present in the crop, followed by the (2) solubilization of the protein crystals, (3) activating the proteins. Then (4) the protein binds to the receptors, and (5) pores form in the membrane and cell lysis occurs (obtained from Schünemann 2012 in Schünemann *et al.* 2014).

Due to its believed target-specificity, based on the specific gut receptors of insects and its basic pH, Bt-based pesticides are considered to not have any adverse effects on non-target organisms. Therefore, and as stated previously, there are a few studies that point possible effects. For example, Dipel® ES demonstrated to have the potential to cause immobilization and mortality of neonates and immobilization of adults of *Daphnia magna*, a non-target organism, at environmentally relevant concentrations (De Souza Machado *et al.* 2017). In mammals, Dipel® administration led to lipid peroxidation and oxidative stress in rat liver at concentrations of 1mg/100g of body mass (Shaban *et al.* 2003).

When it comes to the state of the art concerning the effect of Bt in cell lines, it either concerns non-insecticidal Bt proteins or Bt-incorporated plants, the last has being considered as non-toxic to humans (Betz *et al.* 2000). The number of studies with this biopesticide is scarce. One performed by Thomas and Ellar (1983) showed that non-treated δ-endotoxin crystals did not exerted toxicity in the different *in vitro* and *in vivo* systems, while alkali-solubilized crystal δ-endotoxin caused rapid cytological and cytopathological changes in different animal's cell lines. Another study demonstrated the genotoxic potential of a Bt-based pesticide on adult zebrafish as well as embryo toxic effects, while also demonstrating the possibility that different endotoxins have different levels of effect was observed (Grisolia *et al.* 2009).

Considering this, there is an interest in fulfilling this lack of knowledge, and possible bring new lights to the eventual danger of this biopesticide to biological systems.

1.4 Testing approaches: advantages and disadvantages

Considering the several reported effects of these substances, efficient and practical methods need to be developed to evaluate pesticide effects in biological systems. For non-target organisms, *in vivo* assays are commonly performed, while for cell lines testing, *in vitro* assays are the most popular methods.

1.4.1 *In vivo* approach

In vivo assays are commonly performed to detect contaminants effects, as they are the most direct method to access the effects (Saeidnia *et al.* 2015), while at the same time, different tissues may be tested. Adding to this, the reduced period of growth and reproduction of some model organisms may also be advantageous. However, there are some disadvantages when performing animal tests, such as the high cost, space and labour needed to maintain the organisms (Taju *et al.* 2012). Also, the existence of ethical problems related to the maintenance, well-being and sacrifice of several millions of organisms per year (European Union Commission 2013, Hartung 2008, Doke and Dhawale 2015) have been a point of discussion.

Therefore, there is a growing tendency to take into consideration the 3Rs principle that consists in the: a) *Reduction* of the number of tested organisms, b) *Refinement* of the experiments as reducing the suffering to the minimum, and c) *Replacement* of *in vivo* for alternative methods (Cazarin *et al.* 2004) such as the *in vitro* and *ex vivo* methods (Russell 1957, Doke and Dhawale 2015).

1.4.2 *In vitro* approach

In vitro testing fulfils most of 3R's principles, since there is no animal suffering and sacrifice (Reduction), while at the same time having other advantages when compared to *in vivo* methods, since these studies are optimized to perform, at a faster step, the first evaluation of some contaminants effect (Replacement). Simultaneously, several cell-lines are cost-effectiveness and occupy a reduced amount of space and can be easily adapted to automated high-throughput screening technologies (Taju *et al.* 2012, Fent 2001, Doke and Dhawale 2015). Hence, *in vitro* is very useful to evaluate cellular toxicology, including the toxic modes of action and effects, and to understand the toxicological processes (Fent 2001). On the other hand, there are some limitations associated with *in vitro* experiments: cell-lines do not account for the biokinetics, tissue distribution, bio-transformation, and environmental factors (and processes) that may occur *in vivo* and influence toxicity (Fent 2001), making the extrapolation to corresponding *in vivo* results difficult and variable in some cases (Saeidnia *et al.* 2015, Yoon *et al.* 2014). At the same time, genotoxicity assays in some mammalian cell-lines have demonstrated a high prevalence of

"misleading" positive results, requiring confirmation from *in vivo* and/or other studies (Whitwell *et al.* 2015).

1.4.3 *Ex vivo* as an alternative approach

As an attempt to fulfil the identified gaps, the *ex vivo* method appears as a viable alternative. This method is described by Dusinska *et al.* (2012) as a model between *in vitro* and *in vivo*, where whole tissue slices are cultured, maintaining cytoarchitecture, some intercellular connections, while maintaining metabolic processes with resemblances with the *in vivo* situation. As a possible alternative to *in vivo* testing, it conjugates the advantages of, per example the *in vitro* method, while avoiding the corresponding disadvantages: *ex vivo* method greatly reduces the number of individuals (Reduction) in comparison to *in vivo*, while at the same time tissues can be subject to different treatments. This methodology also reduces the experiment time and space, in size and volume (Refinement), and it may be possibly to use in the future as an alternative to *in vivo* and even to *in vitro* experiments (Replacement) for a faster screening of waterborne contaminants' effects. Notwithstanding, the limitations of the *ex vivo* experiment rely on the limited lifespan of the tissue leading to experiments of short-time expositions, and the resulting response is limited to that tissue and not the full organism (Ong *et al.* 2013).

1.5 DNA as a target molecule of pesticides

DNA is of great importance to primary functions (Alberts *et al.* 2002). Changes occurring in this molecule may have negative consequences, leading to severe problems within the cell, the tissue or even the organism in question (Robertson 2001). As such, a pesticide that exerts negative effects on the integrity of genetic material is considered to have a genotoxic effect, which may lead to several physiologic and morphologic defects (Bolognesi *et al.* 2003, Rahman *et al.* 2002). These genotoxic effects may affect DNA directly, by inducing lesion and/or mutations, or indirectly, by affecting DNA repair enzymes or even cell lyses. Affected cells may end up going through apoptosis, or they may proliferate with damaged DNA, leading to further negative effects (Kirsch-volders *et al.* 2000, Kirsch-volders *et al.* 2003). Therefore, the evaluation of genotoxicity is very relevant, providing additional information about the negative effects of pesticides to non-target organisms.

1.5.1 The tool comet assay

For this purpose, the genotoxic evaluation is a valuable parameter to measure and can be assessed by using the comet assay, as a method to quantify DNA damage. This technique allows

to measure unspecific damage, such as single and double strand breaks, and alkali labile sites, of eukaryotic cells (Azqueta *et al.* 2014). As a complement, this assay may be improved using DNA lesion-specific repair enzymes that reveal specific DNA oxidative damage, increasing the assay's sensibility and specificity. The most used endonucleases are formamidopyrimidine DNA glycosylase (FPG) and endonuclease III (EndoIII), which convert oxidised purines and pyrimidines into breaks, respectively (Azqueta *et al.* 2009).

1.6 Biological models

1.6.1 The crayfish *Procambarus clarkii*

As an example of an environmental non-target organism, *Procambarus clarkii*, the red swamp crayfish, was chosen due to some interesting characteristics, namely their great resistance to non-optimal environmental conditions, which explains their presence in several contaminated areas (López *et al.* 2004, Faria *et al.* 2010). Despite this, *P. clarkii* shows sensibility to environmental changes, thus sustaining the idea of being a good model for ecogenotoxicological studies (López *et al.* 2004, Faria *et al.* 2010, Vioque-Fernández *et al.* 2007). Furthermore, its ability to reproduce at a fast step helps this species to be considered as a well-succeeded one. Epithelial gill cells will be the focus in this study considering that they are, by default, an extensive and constant contact with the aquatic environment (Figueiredo-Fernandes *et al.* 2007). Furthermore, they have a high renovation rate (Ahmad *et al.* 2005), that interfere with the persistence of DNA damage, potentiating the assessment of recent events, which makes gills an excellent target organ to assess genotoxicity. Of the pesticides mentioned before, only a commercial formulation of penoxsulam has been tested in *P. clarkii*, and it demonstrated to be genotoxic to populations previously exposed to the same pesticide (Costa *et al.* 2018).

1.6.2 The cell line HepG2

Immortalized cell lines are commonly used as simple *in vitro* models for research (Kaur and Dufour 2012) of the different effects and processes of a toxic in a cellular perspective (Fent 2001). HepG2 is a cell line first isolated in 1975, from a liver hepatocellular carcinoma of a 15 years old Argentinian male (Aden *et al.* 1979). These are epithelial and adherent cells commonly used as *in vitro* models for human hepatocytes, since they are easy to maintain and keep a lot of specialized functions, such as the secretions of specific proteins and enzymes (Mersch-Sundermann *et al.* 2004). They are commonly used to study liver and xenobiotic metabolism, substances toxicity, genotoxicity and cytotoxicity, as well as for drug targeting studies (Mersch-Sundermann *et al.* 2004, Cederbaum *et al.* 2001, Valentin-Severin *et al.* 2003). Since pesticides

residues can enter the blood stream through dermal contact, the respiratory and digestive system, they may reach the liver (Freire *et al.* 2015, Karami-Mohajeri *et al.* 2017), and may be metabolized. Consequently, this cell line was chosen to mimic the possible outcomes to pesticide exposure, by measuring genotoxicity and cytotoxicity.

1.7 Study framework and objectives

Bearing all these considerations in mind, this study intended to encompass the effect of pesticides in different biological models, using distinct approaches, to minimize the negative impacts of *in vivo* experimental designs. Therefore, the main objectives were: (i) to validate the *ex vivo* technique as an alternative in animal research, (ii) to assess the genotoxicity of three chemically-based pesticides dimethoate (insecticide), imazalil (fungicide) and penoxsulam (herbicide) and the bioinsecticide Turex® to gill cells of *Procambarus clarkii*, using an *ex vivo* approach and also (iii) to disclose the cytotoxicity and genotoxicity, *in vitro*, of Turex® to the cell line HepG2.

2. Material and Methods

2.1 Chemicals

Dimethoate, imazalil and penoxsulam were obtained from Sigma-Aldrich Chemical Company (Spain), and the commercial formulation Turex® was obtained from Biosani (Portugal), containing *Bacillus thuringiensis aizawai* GC-91 (3.80% p/p). DNA lesion-specific repair enzymes (FPG and EndoIII) were purchased from Professor Andrew Collins (University of Oslo, Norway). For cell maintenance, Dulbecco's Modified Eagle Medium (DMEM), trypsin-ethylenediaminetetraacetate (Trypsin-EDTA), fetal bovine serum (FBS), amphotericin B (Fungizone) and sodium pyruvate were purchased from Thermo Fisher Scientific (Waltham, MS, USA). Phosphate-buffered saline (PBS), penicillin-streptomycin (Pen-Strep) and L-Glutamine were obtained from Biochrom (Germany). All the other chemicals used for the comet assay and the MTT test were also obtained from Sigma-Aldrich Chemical Company (Spain).

2.2 Test animals and experimental design

2.2.1 Crayfish collection and laboratory maintenance

Tested organisms, the crayfish *Procambarus clarkii*, were captured from Minho River, Vila Nova de Cerveira (Portugal), a location described as free of pesticides (IST/INAG, 2001; Santos et al. 2013), with an average length of 11.00 ± 0.05 cm. Crayfish were acclimatized in the laboratory for 15 days 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 19 ± 1ºC, pH 7.1 ± 0.3, nitrate 27 ± 0.2 mg L^{-1}, nitrite 0.07 ± 0.02 mg L^{-1}, ammonia 0.2 ± 0.04 mg L^{-1}, and dissolved oxygen 8.2 ± 0.3 mg L^{-1}. In the period leading up to the experiment, crayfish were fed *ad libitum*.

2.2.2 *Ex vivo* approach - crayfish gill cells

Only male crayfish were considered for the *ex vivo* exposure to the chemically-based pesticides. Organisms were divided in groups of n=6 for dimethoate, n=7 for imazalil and n=7 for penoxsulam. Regarding the *ex vivo* exposure to Turex®, both genders were considered, where one group of males (n=8) and one group of females (n=8) were used. After the acclimation period, crayfish were sacrificed by a transection on the posterior side of the rostrum, followed by the removal of the carapace and the extraction of the gills onto a petri dish (one for each animal) with

1.5 mL of chilled PBS, to remove unwanted particles. Then, PBS (1.5 mL) was replaced, and the tissue was minced to release as many cells as possible, to obtain a cellular suspension. After, the cellular suspension was divided equally between microtubes: 7 microtubes per organism in step 1 and 3, and 3 microtubes per organism in step 2. Therefore, 1 microtube was used per organism per condition/time. To avoid cell's precipitation, microtubes were placed in a mechanical rotator, at 0,9 RT minute^{-1}, slightly inclined, during the exposure periods (Fig. 2).

For the DNA integrity evaluation, gill cells suspension was centrifuged at 1500 rpm, for 5 minutes at 4 ºC. The obtained pellet was resuspended with medium (1.5 mL) corresponding to either a negative control (NC) with PBS and a positive control (PC) with EMS dissolved in distilled water (5 mg L^{-1}), and gill cells were exposed *ex vivo* for 2 and 4 hours, while in a mechanic rotator. The positive control EMS (ethyl methanesulfonate), which is widely used as a genotoxic model (Pant *et al.* 2014), was used to validate the comet assay procedure, as well as the suitability of gill's cells.

Then, and to validate the application of this integrative approach in the assessment of the genotoxic potential of each pesticide, microtubes with cellular suspension were as well centrifuged and resuspended with medium (1.5 mL) corresponding to the same NC, and an environmentally realistic concentration of each pesticide: dimethoate (D) (20 µg L^{-1}) (Scoy *et al.* 2016), dissolved in distilled water, imazalil (I) (160 µg L^{-1}) (Castillo *et al.* 2006) and penoxsulam (P) (23 µg L^{-1}) (Rodrigues and Almeida 2005), both dissolved in PBS. Genotoxicity of Turex® (T1 – 25, T2 - 50, T3 - 100, T4 - 200 and T5 - 400 µg L^{-1} in PBS) was also evaluated, all for an exposure period of 2 hours.

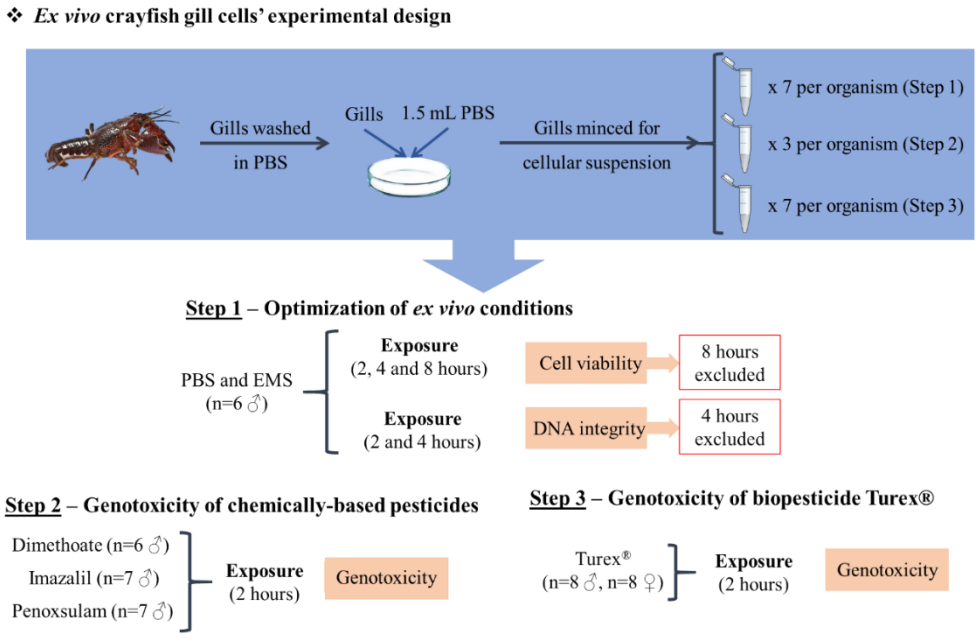

Fig. 2 – Experimental design of the different steps performed with crayfish gill cells, from the extraction of the tissue, to the optimization of the *ex vivo* exposure, and the genotoxicity evaluation of the pesticides.

2.3 Liver cell culture and experimental design

2.3.1 *In vitro* approach - liver cell culture

HepG2 cell line was obtained from European Collection of Authenticated Cell Cultures (ECACC) and supplied by Sigma-Aldrich (Spain). Cells were grown in DMEM medium supplemented with 10% FBS, 2mM L-glutamine, 1 mM sodium pyruvate, 100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, and 250 µg mL^{-1} fungizone, in 25 cm^2 flasks, at 37°C in a 5% CO_2 humidified atmosphere. Subculturing was performed twice a week, once the cells reached about 80% confluency. Cells were then washed carefully with 2.5 mL of PBS, trypsinized with 1 mL of 0.25% trypsin-EDTA, for 5 minutes at 37ºC. Neutralization was achieved adding 2 mL of supplemented DMEM medium, and up and down was performed to individualize cells. Cultivation was concluded in a new flask with 1 mL of cell suspension mixed with 5 to 7 mL of supplemented DMEM medium.

For the viability assessment, cells were seeded at a density of 18×10^4 mL^{-1} in 96-well plates. After 24 h the medium was replaced with either (a) fresh medium (NC), (b) fresh medium containing one of the five concentrations of Turex® (T1 - 250, T2 - 500, T3 - 1000, T4 - 1500 or T5 - 2000 µg L^{-1} in PBS), (c) fresh medium containing one of the five concentrations of Turex® that had a previous activation with 4N NaOH, maintaining pH 10 for one hour (T1B, T2B, T3B, T4B or T5B), and (d) fresh medium with the same quantity of NaOH used previously for each biopesticide concentration (C1, C2, C3, C4 and C5), as NaOH controls, with three technical replicas per condition (Fig. 3).

Concerning the DNA integrity evaluation, cells were seeded at a density of 18×10^4 mL^{-1} in 6-well plates. Procedure and conditions were carried out identically to what was described in cell viability, except for the NaOH control, where only the control for the highest concentration was tested (C5). After the 24 and the 48 hours treatment, each well was washed with 1 mL PBS, trypsinised with 300 µL of 0.25% trypsin-EDTA, for 5 minutes at 37ºC, and 600 µL of fresh medium was added to neutralize it. Cell suspension was then transferred to microtubes, centrifuged at 200g for 5 minutes at 4ºC, followed by the removal of the supernatant, resuspended with 1 mL of PBS, and then recentrifuged. The PC treatment consisted in the resuspension of the cells in 100 µM H_2O_2 for 5 minutes, followed by a centrifugation and resuspension with 0.5 mL of PBS. The rest of the Microtubes were also resuspended with 0.5 mL of PBS.

❖ ***In vitro* liver cells' experimental design**

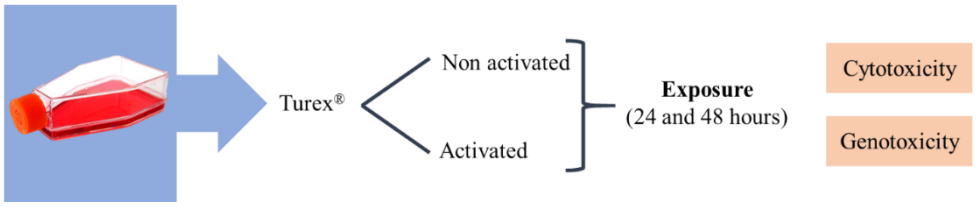

Fig. 3 – Experimental design of the different steps performed with HepG2 cell line, for the assessment of cytotoxicity and genotoxicity of Turex®.

2.4 Cell Viability

For the optimization of the *ex vivo* approach, cell viability of the crayfish cellular suspension in PBS, after 2, 4 and 8 hours, was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay, at a final concentration of 5 mg mL^{-1}, based on Twentyman and Luscombe (1987), with some modifications. Briefly, gill cells were incubated with MTT solution for 4 hours, in the dark, at 25°C. After MTT incubation, cells were submerged in DMSO and mixed thoroughly for approximately 60 seconds (until no purple crystals were left on them). The quantity of produced formazan is proportional to the number of viable cells, and it was measured at 570 nm in a spectrophotometer (SpectraMax 190). The assay results lead to the exclusion of the 8-hour exposure time from the following tests.

Cell viability of HepG2 was measured using the same technique as described above. After cell exposure as detailed previously, 50 µL of MTT (1 mg mL^{-1}) in PBS was added in each well, after 24 and 48 hours, for 4 h at 37 °C in an atmosphere with 5% CO_2. The medium with MTT was then removed and 150 µL of DMSO was added to each well, and the plate was placed in an orbital shaker for 2 hours. Viability was then measured as described above. In every condition of exposure, it was demonstrated that cells had viability of >70% (Tice *et al.* 2000), therefore, all conditions were tested for its potential genotoxic effects.

2.5 Evaluation of genetic damage

The conventional alkaline version of the comet assay was performed according to the methodology of Collins (2004) as adapted by Guilherme *et al.* (2010), with the proper adjustments to assay procedure. For the assessment of the pesticides' genotoxicity (dimethoate, imazalil and penoxsulam), an extra step of digesting the nucleoids with endonucleases was added. A system of eight or ten gels per slide was adopted, based on a model created by Shaposhnikov *et al.* (2010), to increase the assay output. Thus, crayfish cellular suspensions were centrifuged, and the

supernatant was taken out, leaving 10 µL inside the microtube, to which 30 µL of low melting point agarose (1% in PBS) was added. Eight or ten drops with 6 µL of cell suspension were placed onto the precoated slide (with 1% normal melting point agarose), without coverslips, containing each gel approximately 1500 cells. Gels were left for ± 5 min at 4 °C, to solidify agarose, and then immersed in a lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 1% Triton X-100, pH 10) at 4 °C, for 1 hour. After lysis of agarose-embedded cells, slides were washed 3 times with buffer (0.1 M KCl, 0.5 mM EDTA, 40 mM HEPES, 0.2 mg mL^{-1} bovine serum albumin, pH 8) at 4 °C.

For the chemically-based pesticides, three sets of slides were prepared: first set was incubated with (1) FPG, second with (2) EndoIII, which converts oxidized purines and pyrimidines into DNA single strand breaks, respectively (Azqueta *et al.* 2009). A third (3) set was incubated only with buffer. Hence, 30 µL of each enzyme (diluted in buffer) was applied in each gel, along with a coverslip, prior to incubation at 37 °C for 30 minutes in a humidified atmosphere. As for the Turex® treatment, only one set of slides were prepared, that were kept in the lysis solution until the next step. The slides were then gently placed in the electrophoresis tank, immersed in electrophoresis solution (± 20 min) for alkaline treatment. DNA migration was performed for crayfish cell at a fixed voltage of 25 V, a current of 300 mA which results in 1.1 V cm^{-1} (achieved by adjusting the buffer volume in the electrophoresis tank). The slides were stained with ethidium bromide (20 g mL^{-1}).

Fifty nucleoids were observed per gel, using a Leica DMLS fluorescence microscope (x400 magnification). The DNA damage was quantified by visual classification of nucleoids into five comet classes, according to the tail intensity and length, from 0 (no tail) to 4 (almost all DNA in tail) (Collins 2004). The total score expressed as a genetic damage indicator (GDI) was calculated multiplying the percentage of nucleoids in each class by the corresponding factor, according to this formula:

$$\mathrm{GDI} = \sum \%\ \text{nucleoids class}\ i\ \times i$$

where *i* is the number of each defined class (ranging within 0-4) and GDI values were inherently expressed as arbitrary units in a scale of 0–400 per 100 scored nucleoids. The difference between GDI_{FPG} and GDI, resulting in the FPG-associated net enzyme-sensitive sites (NSS_{FPG}), as well as between $GDI_{EndoIII}$ and GDI, resulting in the EndoIII-associated net enzyme-sensitive sites ($NSS_{EndoIII}$) were calculated to indicate additional DNA breaks, which occur in net enzyme-sensitive sites solely (Azqueta et al. 2009). Moreover, the frequency of nucleoids observed in each comet class considering GDI, GDI_{FPG} and $GDI_{EndoIII}$ was also expressed, as recommended by Azqueta *et al.* (2009).

In what concerns to the HepG2 cell line, the comet assay was performed as explained above with the following differences: 50 µL of each cell suspension was mixed with 50 µL of low melting point agarose (1% in PBS), instead of the 10-30 ratio used with crayfish gill cells, and electrophoresis took place at a fixed voltage of 17 V, a current of 300 mA which resulted in 0.7 V cm^{-1} for 30 minutes.

2.6 Statistical analysis

Statistica 7.0 software was used for statistical analysis. All data was first tested for normality and homogeneity of variance, and transformed if necessary, to meet statistical demands. One-way Analysis of Variance (ANOVA), followed by Tukey HSD test as *Post-hoc* comparison, was applied to comparations between control groups of different times of the *ex vivo* exposure, and between groups of different concentrations of Turex®. One-way Analysis of Variance (ANOVA), followed by Dunnett test as *Post-hoc* comparison, was performed for the assessment of differences between treated groups and the respective negative control, within the same exposure duration, for the *ex vivo* exposure. For comparations between the negative and positive control groups in the *in vitro* exposure, the independent-samples *t*-test was applied. In all the analyses, differences between means were considered significant when $p<0.05$ (Zar 1996).

3. Results

3.1 Crayfish gill cells' *ex vivo* approach

For the validation of the exposure periods of the *ex vivo* method, cell viability and DNA integrity were assessed.

3.1.1 Step 1 – Optimization of *ex vivo* conditions

3.1.1.1 Cell viability

Table II. Cell viability (%) of crayfish gill cells in PBS (negative control – NC), measured by the MTT assay, after 2, 4 and 8 hours. Black values are above 70% viability, while red values are below the acceptance limit.

Exposure Conditions		Cell viability (%)
NC	2h	70.3
	4h	77.9
	8h	66.9

Viability analyses demonstrated that cells had an acceptable percentage viability of above 70% (Tice *et al.* 2000) for most of the *ex vivo* experiment, except for the 8-hours' exposure. Therefore, this exposure time was excluded from the following tests, while the 2 and 4 hours of exposure will be tested for genotoxicity in step 2.

3.1.1.2 Evaluation of DNA integrity

Non-specific DNA damage

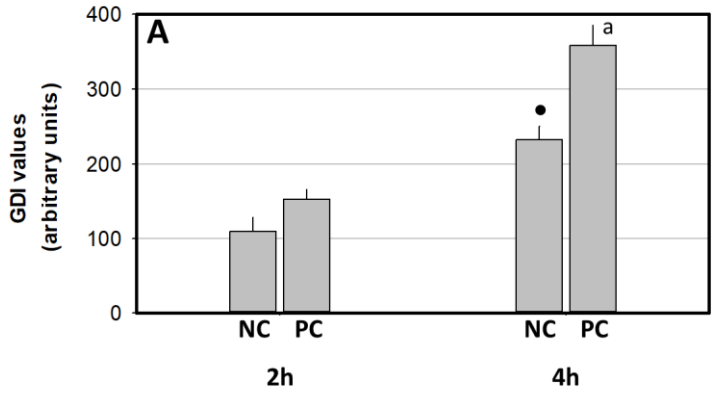

Fig. 4 - Mean values of DNA damage, measured by comet assay in gill cells of *Procambarus clarkii* exposed to 5 mg L^{-1} EMS (positive control – PC), during 2 and 4 hours. NC (PBS) was the negative control group. The genetic damage indicator (GDI) was measured by the standard (alkaline) comet assay. Bars represent the standard error. Statistically significant differences ($p < 0.05$) are represented as: (a) in relation to negative control (NC), within the same exposure time; (●) between NC groups of both exposure times.

The results of the GDI parameter (reflecting an unspecific DNA damage), in what concerns to the progression of the baseline DNA damage (Fig. 4), demonstrated a decrease of the DNA integrity in the NC group after 4 hours. In what concerns the PC group (Fig. 4), after 4h it presented a significant increase in DNA damage, in comparison with the NC group.

Specific oxidative DNA damage

The detection of oxidized bases was achieved by the comet assay with an extra step where nucleoids were incubated with the DNA lesion-specific repair enzymes EndoIII and FPG (Fig. 5 and 6).

FPG associated damage

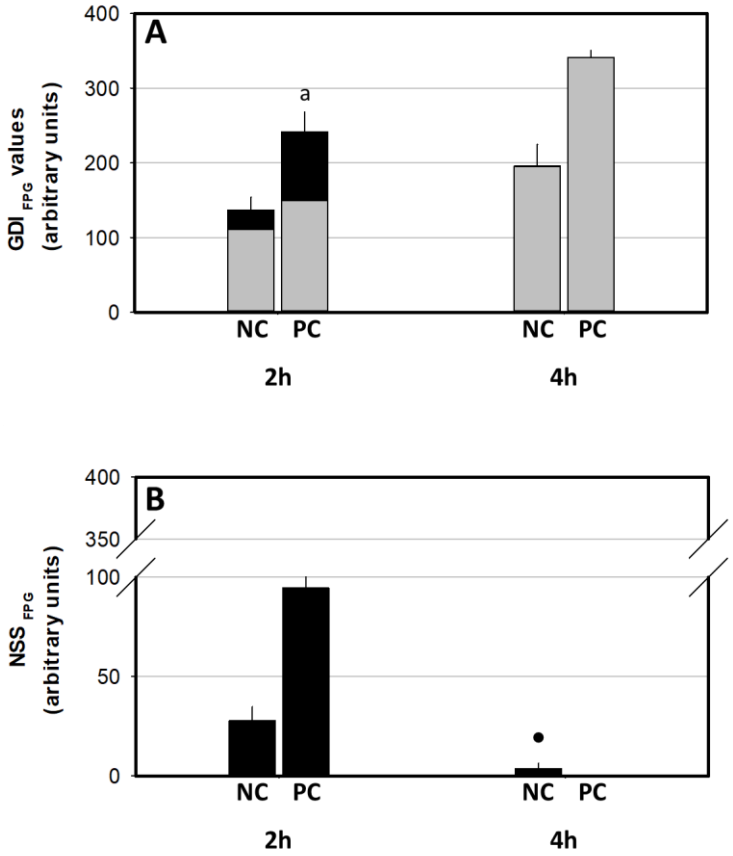

Fig. 5 - Mean values of DNA damage, measured by comet assay in gill cells of *Procambarus clarkii* exposed to 5 mg L^{-1} EMS (positive control – PC), during 2 and 4 hours. NC (PBS) was the negative control group; (A) overall damage (GDI_{FPG}) and partial scores, namely genetic damage indicator (GDI; grey) and additional DNA breaks corresponding to net FPG-sensitive sites (NSS_{FPG}; black); (B) NSS_{FPG} alone. Bars represent the standard error. Statistically significant differences ($p < 0.05$) are represented as: (a) in relation to negative control (NC), within the same exposure time; (●) between NC groups of both exposure times.

In the GDI_{FPG} parameter, the positive control group increased the DNA damage significantly, when compared to the NC group, after 2 hours (Fig. 5A). The NSS_{FPG} parameter (Fig. 5B) displayed a significantly lower level of specific DNA damage in the NC group, after 4-hour exposure, when compared to the other exposure time.

EndoIII associated damage

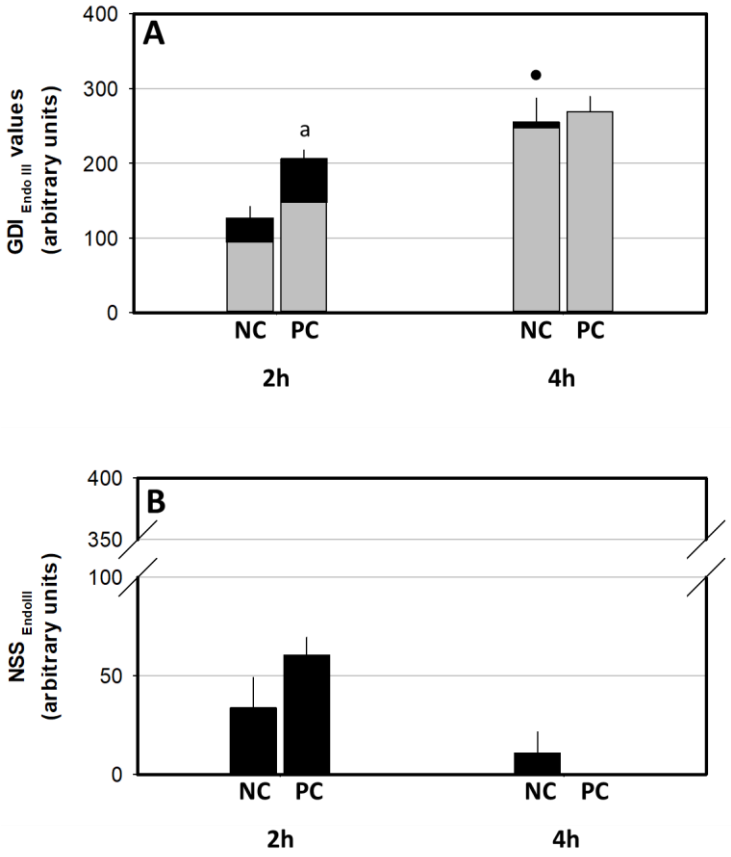

Fig. 6 - Mean values of DNA damage, measured by comet assay in gill cells of *Procambarus clarkii* exposed to 5 mg L^{-1} EMS (positive control – PC), during 2 and 4 hours. NC (PBS) was the negative control group; (A) overall damage ($GDI_{EndoIII}$) and partial scores, namely genetic damage indicator (GDI; light grey) and additional DNA breaks corresponding to net EndoIII-sensitive sites ($NSS_{EndoIII}$; dark grey); (B) $NSS_{EndoIII}$ alone. Bars represent the standard error. Statistically significant differences ($p < 0.05$) are represented as: (a) in relation to negative control (NC), within the same exposure time; (●) between NC groups of both exposure times.

Towards assessing the integrity of the DNA during the experiment period, differences between were evaluated (Fig. 6A). Thus, in the $GDI_{EndoIII}$ parameter (Fig. 6A), it was possible to observe that NC group of the 4-hour moment presented higher values when compared to the group which represents the first moment of exposure (2-hour). Concerning the 2nd hour of exposure, the PC group displayed significantly higher DNA damage than the negative control (NC), which was not observed after 4 hours of exposure. In what concerns to the $NSS_{EndoIII}$ parameter (Fig. 6B), no significant differences were found.

3.1.2 Step 2 – Genotoxicity of chemically-based pesticides

After the *ex vivo* approach optimization, the 2-hour exposure was elected. The validation of this approach was performed by testing the genotoxic effect of three chemically-based pesticides, from the most used classes: the insecticide dimethoate, the fungicide imazalil, and the herbicide penoxsulam.

Non-specific DNA damage

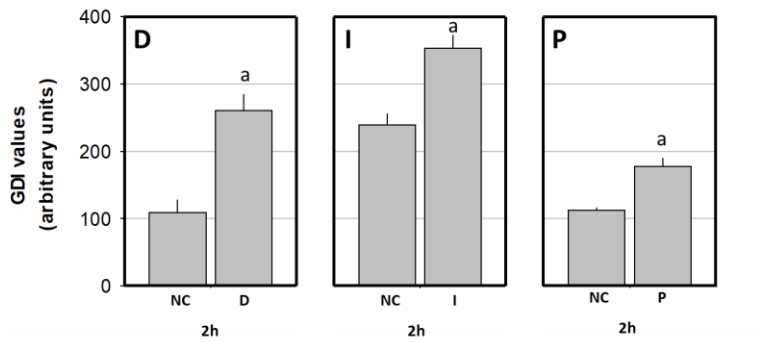

Fig. 7 - Mean values of DNA damage, measured by comet assay in gill cells of *Procambarus clarkii* exposed to 20 µg L^{-1} of dimethoate (D), to 160 µg L^{-1} of imazalil (I) and to 23 µg L^{-1} of penoxsulam (P), for 2 hours. NC (PBS) was the negative control group. The genetic damage indicator (GDI) was measured by the standard (alkaline) comet assay. Bars represent the standard error. Statistically significant differences ($p < 0.05$) are represented as (a) in relation to negative control (NC), within the same exposure time.

The results for the unspecific DNA damage (GDI) demonstrated that the all tested pesticides dimethoate (D), imazalil (I) and penoxsulam (P), increased significantly the DNA damage levels, when compared to their corresponding negative controls (NC) (Fig. 7).

Table III. Mean frequencies (%) of damaged nucleoids classes (± standard error), measured by the comet assay, in gill cells of *Procambarus clarkii* exposed to PBS (negative control - NC), 20 µg L^{-1} of dimethoate (D), 160 µg L^{-1} of imazalil (I) and 23 µg L^{-1} of penoxsulam (P), for 2 hours. Statistically significant differences ($p<0.05$) are represented as (a) versus NC within the same exposure time.

Exposure Conditions		GDI DNA Damage Classes					
		0	1	2	3	4	Sub-total (2+3+4)
2h	NC	19.30±6.49	54.36±7.14	24.00±10.49	2.33±1.67	0.00±0.00	26.33±12.16
	D	1.06±0.61[a]	22.61±5.00[a]	16.11±4.89	35.50±3.20[a]	24.72±10.13[a]	76.33±18.22[a]
	NC	0.00±0.00	9.57±2.13	47.86±9.98	36.14±9.23	6.43±3.80	90.43±23.01
	I	0.00±0.00	0.20±0.17[a]	3.40±1.45[a]	39.00±13.02	57.40±14.43[a]	99.80±28.90
	NC	0.00±0.00	87.42±2.85	12.40±2.71	0.17±0.17	0.00±0.00	12.58±2.88
	P	0.00±0.00	34.23±9.84	53.65±10.08[a]	12.12±4.85[a]	0.00±0.00[a]	65.77±14.93

To perform a more detailed analysis of how DNA damage varied over tested conditions, the damage classes of GDI were analysed individually. It was possible to observe different patterns (Table III). One is related to the NC group related to dimethoate (D) and penoxsulam (P) groups, where class 1 was most frequent, while the NC group of imazalil (I) had classes 2 and 3 as predominant. Another pattern was that D and I groups had as most frequent classes 3 and 4, which is reflected in the sub-total values. When it comes to P group, unlike the other two pesticides, it demonstrated as most common classes 1 and 2, like its NC group common classes. Nevertheless, P group had higher DNA damage compared to the NC, highlighted by the sub-total value.

Specific oxidative DNA damage

FPG associated damage

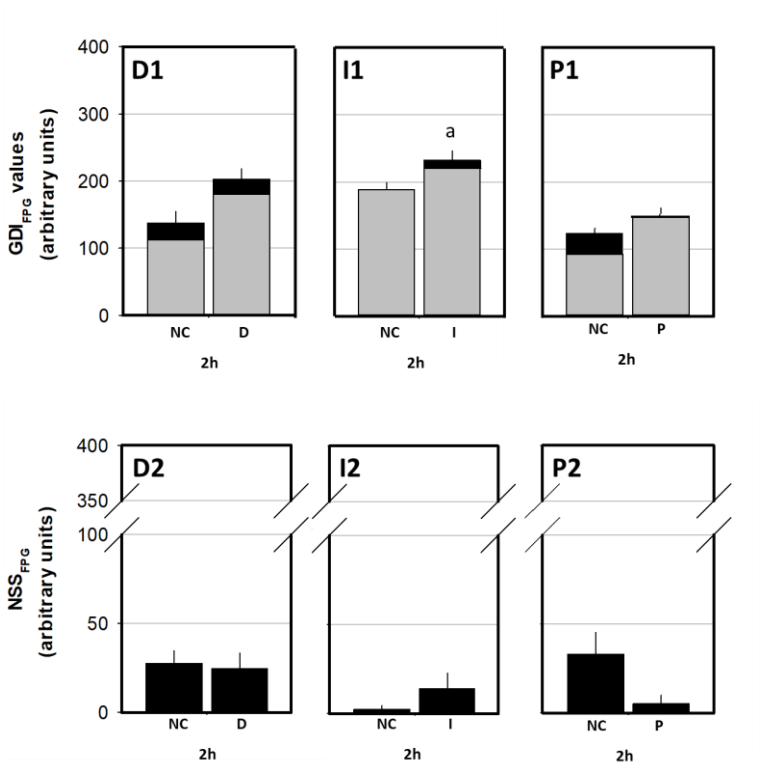

Fig. 8 - Mean values of DNA damage, measured by comet assay in gill cells of *Procambarus clarkii* exposed to 20 µg L^{-1} of dimethoate (D) (graph D1 and D2), to 160 µg L^{-1} of imazalil (I) (graph I1 and I2) and to 23 µg L^{-1} of penoxsulam (P) (graph P1 and P2) , for 2 hours. NC (PBS) was the negative control group; (A) overall damage (GDI_{FPG}) and partial scores, namely genetic damage indicator (GDI; grey) and additional DNA breaks corresponding to net FPG-sensitive sites (NSS_{FPG}; black); (B) NSS_{FPG} alone. Bars represent the standard error. Statistically significant differences ($p < 0.05$) are represented as (a) in relation to negative control (NC), within the same exposure time.

Concerning the GDI_{FPG} parameter, after 2 hours, only the imazalil group showed a significantly higher DNA damage, when compared to the corresponding NC group (Fig. 8A). The NSS_{FPG} parameter (Fig. 8B) did not displayed any significant differences.

Table IV. Mean frequencies (%) of damaged nucleoids classes (± standard error), measured by the comet assay with and extra step of incubation with the FPG, in gill cells of *Procambarus clarkii* exposed to 20 µg L^{-1} of dimethoate (D), 160 µg L^{-1} of imazalil (I) and 23 µg L^{-1} of penoxsulam (P), for 2 hours. NC (PBS) was the negative control group. Statistically significant differences ($p<0.05$) are represented as (a) versus NC within the same exposure time.

Exposure Conditions		GDI_{FPG} DNA Damage Classes					
		0	1	2	3	4	Sub-total (2+3+4)
2h	NC	6.33±4.77	57.00±7.93	30.33±8.72	6.00±3.58	0.33±0.33	36.67±12.64
	D	1.50±0.96[a]	28.00±12.36	39.50±8.54	28.50±9.64	2.50±1.50	70.50±19.68
	NC	0.00±0.00	30.29±4.97	51.86±2.02	16.57±4.99	1.29±0.52	69.71±7.53
	I	0.00±0.00	24.29±7.43	30.29±8.15[a]	34.61±8.24	10.82±4.02[a]	75.71±20.41
	NC	0.33±0.33	76.80±6.81	21.79±6.48	1.07±0.75	0.00±0.00	22.87±7.23
	P	0.00±0.00[a]	56.20±9.87	38.58±8.11[a]	5.22±2.05	0.00±0.00	43.80±10.16[a]

The analysis of the damage classes of GDI_{FPG} (Table IV) presented all the NC groups with the same classes as frequent (1 and 2), while D and I groups demonstrate similar predominant classes 1, 2 and 3. Concerning the P group, the classes pattern resembles what was observed in GDI, presenting classes 1 and 2 as predominant. D and P groups demonstrated higher sub-total values, when compared to the corresponding NC groups.

EndoIII associated damage

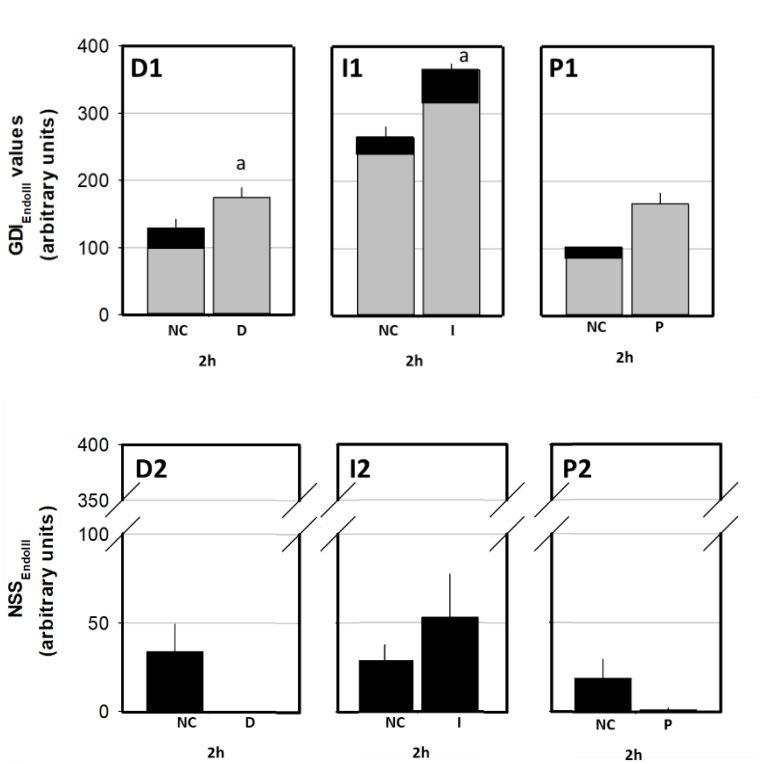

Fig. 9 - Mean values of DNA damage, measured by comet assay in gill cells of *Procambarus clarkii* exposed to 20 µg L^{-1} of dimethoate (D) (graph D1 and D2), to 160 µg L^{-1} of imazalil (I) (graph I1 and I2) and to 23 µg L^{-1} of penoxsulam (P) (graph P1 and P2), for 2 hours. NC (PBS) was the negative control group; (A) overall damage ($GDI_{EndoIII}$) and partial scores, namely genetic damage indicator (GDI; light grey) and additional DNA breaks corresponding to net EndoIII-sensitive sites ($NSS_{EndoIII}$; dark grey); (B) $NSS_{EndoIII}$ alone. Bars represent the standard error. Statistically significant differences ($p < 0.05$) are represented as (a) in relation to negative control (NC), within the same exposure time.

Concerning the $GDI_{EndoIII}$ parameter, it was possible to observe that, after 2 hours, only dimethoate and imazalil groups inflicted a significantly DNA damage, when compared with NC group (Fig. 9A). In what concerns to the $NSS_{EndoIII}$ parameter (Fig. 9B), no significant differences were found.

Table V. Mean frequencies (%) of damaged nucleoids classes (± standard error), measured by the comet assay with an extra step of incubation with the EndoIII, in gill cells of *Procambarus clarkii* exposed to 20 µg L^{-1} of dimethoate (D), 160 µg L^{-1} of imazalil (I) and 23 µg L^{-1} of penoxsulam (P), for 2 hours. NC (PBS) was the negative control group. Statistically significant differences ($p<0.05$) are represented as (a) versus NC within the same exposure time.

Exposure Conditions		$GDI_{EndoIII}$ DNA Damage Classes					
		0	1	2	3	4	Sub-total (2+3+4)
2h	NC	10.67±3.29	55.33±7.28	30.33±9.94	3.67±2.09	0.00±0.00	34.00±12.03
	D	1.20±0.80 [a]	38.50±8.44	45.21±9.25	14.69±6.84 [a]	0.40±0.40	60.30±16.49
	NC	0.00±0.00	7.57±3.31	33.86±7.77	45.43±8.77	13.14±4.33	92.43±20.87
	I	0.00±0.00	0.00±0.00	1.12±0.54	32.67±8.17	66.20±8.57 [a]	100.00±17.28
	NC	0.50±0.22	98.18±0.47	1.32±0.55	0.00±0.00	0.00±0.00	1.32±0.55
	P	0.00±0.00	49.97±10.61	33.93±6.04	16.11±5.47	0.00±0.00	50.03±11.51

When analysing the DNA damage classes individually (Table V), all the NC groups presented the same pattern that the one observed in GDI (class 1 for D and P groups, and classes 2 and 3 for I group). Concerning the pesticides, dimethoate and penoxsulam groups had similar classes as most frequents, 1 and 2, but presented higher sub-total values when compared to the corresponding NC groups. On the other hand, the imazalil group had classes 3 and 4 as predominant.

3.2 Turex® effects

The biopesticide Turex® was tested in an *ex vivo* approach, using crayfish gill cells, and in an *in vitro* approach, with a HepG2 cell line.

3.2.1 Crayfish gill cells' *ex vivo* approach (Step 3)

Non-specific DNA damage

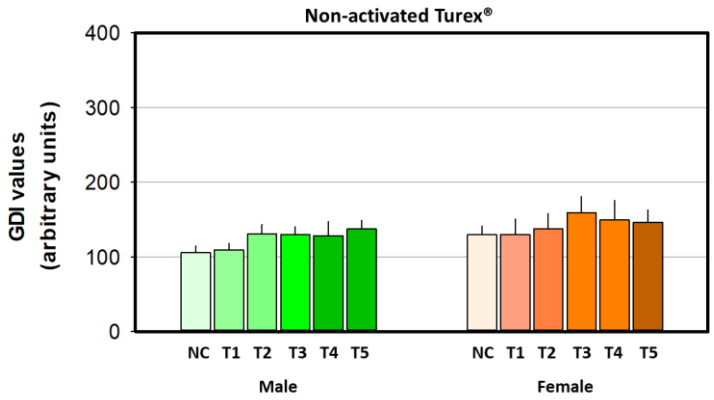

Fig. 10 - Mean values of DNA damage, measured by comet assay in gill cells of males (green) and females (orange) *Procambarus clarkii* exposed to Turex® (T1 - 25, T2 - 50, T3 - 100, T4 - 200 or T5 - 400 µg L^{-1}) for 2 hours. NC (PBS) was the negative control group. The genetic damage indicator (GDI) was measured by the standard (alkaline) comet assay. Bars represent the standard error. Statistically significant differences ($p < 0.05$) are represented as (a) in relation to negative control (NC), within the same exposure time.

Considering the GDI parameter (Fig. 10), no significant DNA damage was observed for all the five concentrations of Turex®, considering both genders of crayfish.

3.2.2 HepG2 cells culture *in vitro* approach

3.2.2.1 Cytotoxic effect

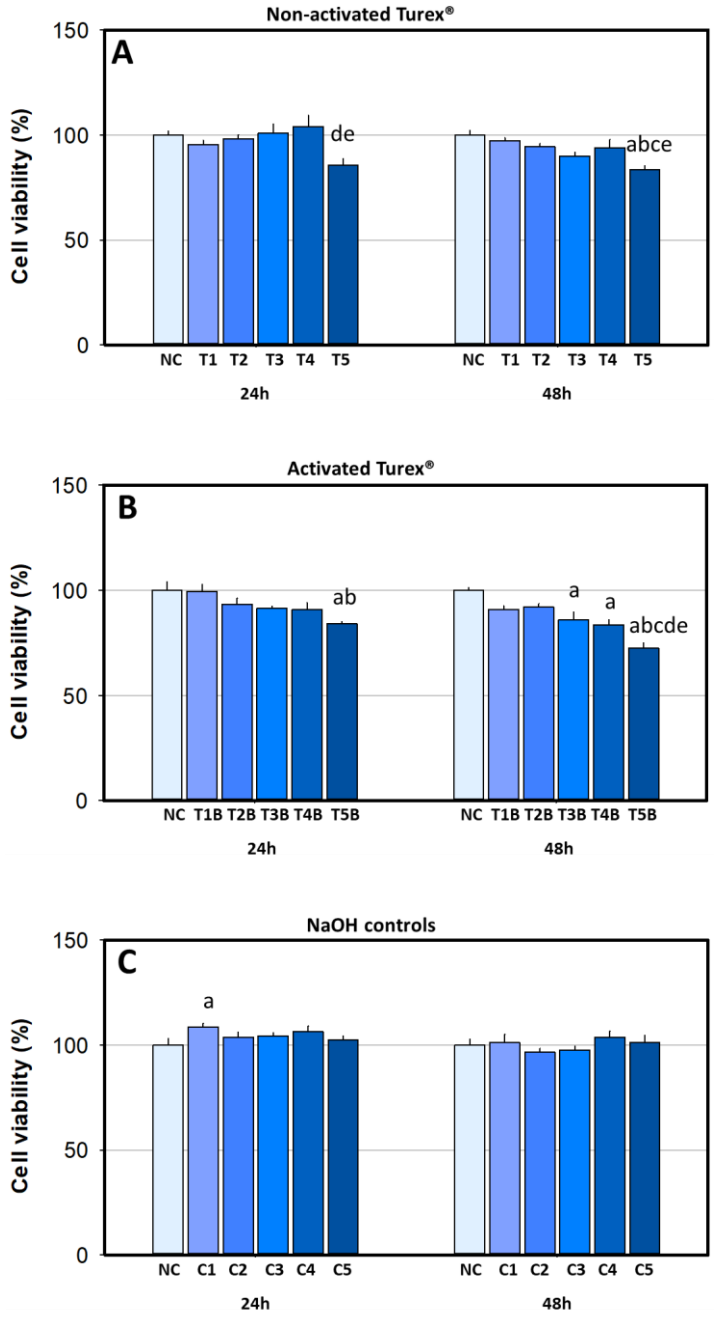

Fig. 11 - Cell viability (%) of HepG2 cells, measured by the MTT assay, after 24 and 48-h exposure to: Turex® (T1 - 250, T2 - 500, T3 - 1000, T4 - 1500 or T5 - 2000 µg L^{-1}), the same concentrations of Turex® that had a previous activation with NaOH, for one hour (T1B, T2B, T3B, T4B or T5B), and controls of NaOH, with corresponding concentrations to the ones used with each pesticide (C1, C2, C3, C4 and C5). NC (PBS) was the negative control group. (A) Exposure to non-activated Turex®; (B) Exposure to Turex® with pre-activation with 4N NaOH; (C) Exposure to 4N NaOH controls. Bars represent the standard error. Statistically significant differences ($p < 0.05$) are represented as (a) in relation to negative control (NC), (b)

in relation to T1, (c) in relation to T2, (d) in relation to T3, and (e) in relation to T4, all within the same exposure time.

The determination of the Turex® effect in the viability of HepG2 demonstrated that the non-activated pesticide group, specifically its highest concentration (T5), only had a significant cytotoxic action when compared to the NC group, after 48 hours (Fig. 11A). Moreover, T5 group was significantly different from T3 and T4 groups, after 24 hours, and from T1, T2 and T4 groups after 48 hours of exposure (Fig. 11A). Activated Turex® groups, with the NaOH treatment (T1B to T5B conditions), demonstrated similar results to the 24 hours. However, the highest concentration (T5B) group was able to induce a higher level of damage, when compared to the NC and to the lowest concentration (T1B) groups, after 24 hours. After 48 hours, all concentrations except for the lowest concentrations (T1B and T2B) groups demonstrated a significant effect compared to the NC groups (Fig. 11B). Adding to this, the T5B group demonstrated again, after 48 hours, to be able to induce a significant higher damage than all the other exposure groups (Fig. 11B). Concerning the effect of the added NaOH group, it was demonstrated that it had no cytotoxic effect, independent of the concentration used and at both times of exposure (Fig. 11C).

3.2.2.2 Evaluation of DNA integrity

Non-specific DNA damage

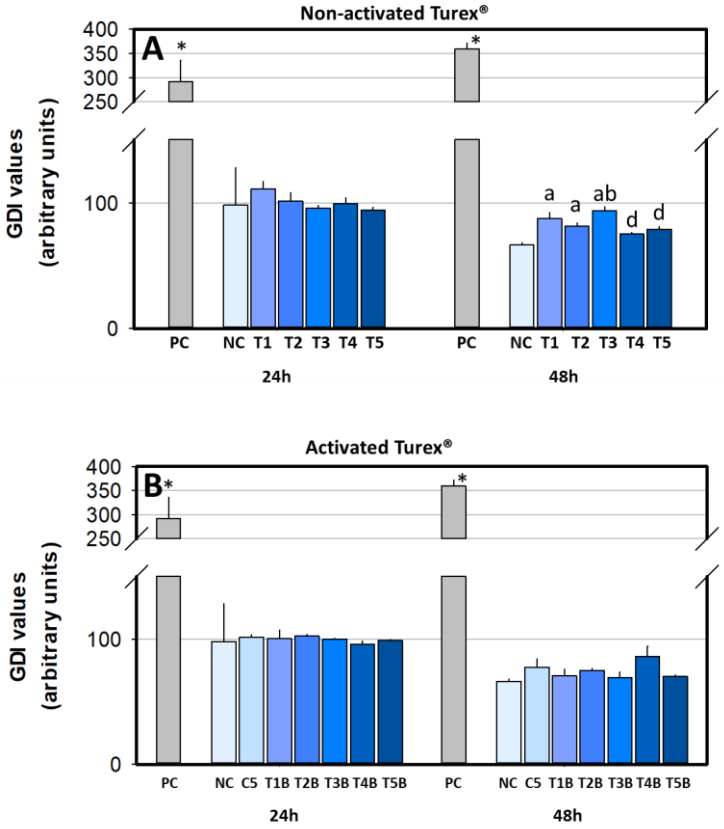

Fig. 12 - Mean values of DNA damage, measured by comet assay in HepG2 cells after exposure of 24 and 48-h exposure to: H_2O_2 (100 µM) as a positive control (PC - grey), Turex® (T1 - 250, T2 - 500, T3 - 1000, T4 - 1500 or T5 - 2000 µg L^{-1}), the same concentrations of Turex® that had a previous activation with added 4N NaOH, maintaining pH 10 for one hour (T1B, T2B, T3B, T4B or T5B), and a control of 4N NaOH, with the corresponding concentration of NaOH applied to T5 (C5). NC (PBS) was the negative control group. The genetic damage indicator (GDI) was measured by the standard (alkaline) comet assay. (A) Exposure to non-activated Turex®; (B) Exposure to Turex® with pre-activation with 4N NaOH and to a NaOH control. Bars represent the standard error. Statistically significant differences ($p < 0.05$) are represented as (a) in relation to negative control (NC), (b) in relation to T1, (c) in relation to T2, and (d) in relation to T3, all within the same exposure time.

The results of the GDI parameter, demonstrated that the PC group had significantly higher damage than the NC group after 24 and 48 hours (Fig. 12A). Concerning the exposure with non-activated Turex®, no significant differences where observed between treatments after 24 hours of exposure (Fig. 12A). After 48 hours, non-activated T1, T2 and T3 groups demonstrated a decrease of the DNA integrity when compared to the NC group (Fig. 12A). Adding to this, it was observed that the T3 group provoked significantly higher DNA damage when compared to T1, T4 and T5

groups (Fig. 12A). In what concerns the exposure with Turex® with previous activation (Fig. 12B), no significant DNA damage was observed.

4. Discussion

4.1 The e*x vivo* approach validation

There are several problems concerning classical toxicological experiments, related to the excessive use of animals and their well-being (e.g. *in vivo* methods), which has been criticised by the scientific and ethics commissions (Festing and Wilkinson 2007, Doke and Dhawale 2015), as well as the difficulty of data extrapolation to infer effects on humans. As an attempt to minimize this problem, the 3 R's (Reduction, Refinement and Replacement) politic has become popular and several efforts have been made to implement it. As already mentioned, and bearing this information in mind, the present study intended to validate the use of the *ex vivo* technique in gill cells of *P. clarkii*, assessing cell viability, using the MTT test, followed by determining DNA integrity, using the comet assay. Then, this approach was applied in the evaluation of the genotoxic effect of three chemically-based pesticides, the insecticide dimethoate, the fungicide imazalil and the herbicide penoxsulam.

Regarding the validation of the *ex vivo* approach, the MTT viability results pointed to the exclusion of the 8th hour of exposure (since only 67% of cells were viable) as it was below the threshold of acceptance of 70% viability, defined by Tice *et al.* (2000). Cells belonging to 2- and 4-hours' sets presented viabilities of 70% and 78%, respectively (table II). Consequently, these exposure times were then evaluated regarding DNA integrity.

Therefore, and to determine how the DNA integrity of gill cells in a cellular suspension would be affected over time, the comet assay was performed with the previously determined exposure times. According to the literature, only a few other studies have used gills in an *ex vivo* context (Beijer *et al.* 2010, Beijer *et al.* 2013), however, none of these studies clarified the repercussions related to the experiment length, and the evaluation of the DNA integrity progression, concerning the *ex vivo* specific conditions. Mentioned studies also demonstrated gills suitability (despite using only filaments) for *ex vivo* assays, giving support to the present research. Moreover, it was also demonstrated that gills could respond to a known genotoxic stimulus (EMS, used as a positive control), confirming the usefulness of this experimental model, while giving a strong contribute to decrease the number of organisms used in toxicological studies.

Concerning the DNA damage induced EMS, known to be a strong genotoxic model (Cavas 2011, Pant *et al.* 2014), it was curios that it did not show a genotoxic effect concerning for non-

specific DNA damage values, after 2 hours, considering the GDI parameter (figure 4). This fact could be related to the possible metabolization time/mode of action requested by the mentioned compound, since its genotoxicity became evident immediately after the 4th hour (figure 5 and 6). Nevertheless, parameters including the use of DNA specific lesion-repair enzymes ($GDI_{EndoIII}$ and GDI_{FPG}) pointed the EMS genotoxicity immediately after the 2nd hour, stating the pro-oxidant potential of this compound (Ansari *et al.* 2011).

It was also observed, for almost all the parameters, that the treated group (PC) assumed values too high (near the roof value), after 4-hours, demonstrating another clear limitation related to experiments with a 4-hours length. Therefore, when using this approach with the comet assay as end point, EMS may have not been the appropriate choice as a positive control. Adding to this, and upon the results for the PC group after 2 hours, it is suggested that for posterior experiments that performed the *ex vivo* approach, a different positive control should be used, for example hydrogen peroxide, also commonly used as a genotoxicant model (Benhusein *et al.* 2010, Nousis *et al.* 2005).

Concerning the DNA damage levels presented by the negative control (NC; PBS), the increased values for the 4-hour NC group, when compared with the 2-hour, demonstrated its unsuitability for this kind of studies, since there was a significant loss of DNA integrity. Therefore, only the 2-hour exposure showed to be suitable for this *ex vivo* experiment and was considered for the study continuation.

4.1.1 Pesticides genotoxic effects

Once selected the 2-hours length as optimal for the *ex vivo* exposure with crayfish gill cells, and by using the comet assay as endpoint, the approach functionality was assessed by determining the genotoxicity of waterborne chemically-based pesticides (insecticide dimethoate, fungicide imazalil and herbicide penoxsulam), using environmental realistic concentrations. Considering this thematic, and to the author best knowledge, there were no studies concerning dimethoate and imazalil negative effects, including genotoxicity, to crayfish, and the present one intended to fulfill this gap. On the other hand, a commercial formulation of penoxsulam has been tested in *P. clarkii*, and it only demonstrated to be genotoxic to populations that have had an history of pesticide-exposure (Costa *et al.* 2018).

Thereafter, the analysis of the 2-hour of exposure demonstrated the genotoxic potential of dimethoate, expressed by both non-specific damage and EndoIII-associated damage parameters (figure 7 and 9). The exposed cells presented low levels of oxidative damage, especially when using EndoIII. This may imply a level of damage induced by dimethoate in pyrimidines that is enough to induce some type of repair mechanism, capable of reducing the DNA damage to levels under the NC, highlighting a different pattern of oxidation considering purines and pyrimidines. One method that could be used to help further understanding the possible DNA repair ability is

the *in vitro* base excision repair (BER) (Azqueta *et al.* 2013). This method, combined with the comet assay, consists in a DNA substrate that contains specific lesions being incubated with a cell extract prepared from the tissue under analysis. The consequent accumulation of breaks is then measured by the comet assay (Azqueta *et al.* 2013, Marques *et al.* 2014), reflecting the repair mechanism. Despite both NSS not demonstrating a significant response, the possibility of dimethoate exerting oxidative damage to purines was not excluded.

The individual analysis of the DNA damage classes demonstrates that the NC group maintained the same predominant class (class 1) in all the parameters, while dimethoate group presented different predominant classes in each parameter. In the non-specific damaged classes class 3 was predominant (table III), while for the FPG-associated damaged classes, class 2 was predominant (table IV), and for the EndoIII-associated damaged classes, class 1 and 2 were more frequent (table V). Adding to this, the sub-total values of the D group demonstrated almost double the a.u. values when compared to the NC group, in every parameter, highlighting the increase of DNA damage induced by dimethoate.

The fungicide imazalil was the only pesticide that demonstrated a genotoxic effect, considering both non-specific and oxidative DNA damage, when both FPG and EndoIII were considered (figure 7, 8 and 9). Even though there is a lack of studies concerning the genotoxic effect of imazalil in organisms related to the crayfish, there are several studies on the effect of this fungicide in human cells. The results presented here are in accordance with what has been assessed by the study of Vindas *et al.* (2004), where all tested concentrations (including the ones of the present study) of this pesticide induced a genotoxic effect after 30 minutes in human lymphocytes, also measured by the comet assay. Also, in lymphocytes, Şişman and Türkez (2010) observed that higher concentrations of imazalil increased the frequency of structural chromosomal aberrations and the rates of micronucleus, again, in agreement with other studies that demonstrate imazalil genotoxicity (Türkez and Aydin 2012, Türkez *et al.* 2012). Even though these cell types are not the same as the ones tested in this study, the general idea that imazalil is capable of provoking DNA damage in a short period of time, is sustained by these studies and by the results obtained when crayfish gill cells were tested.

In what concerns the display of the DNA damage classes separately, it is possible to observe that the NC had a similar predominant class (class 2) in both the non-specific damage classes and FPG-associated damaged classes (table III and IV). The predominant class in the EndoIII-associated damaged classes was class 3 (table V). Imazalil had as predominant class 4, in both non-specific damage classes and EndoIII-associated damaged classes, while for FPG-associated damaged classes, classes 2 and 3 were predominant. In addition, the DNA's integrity in the NC group demonstrated to be affected when compared to other NC groups (also observed in the graphs). This may indicate an interference that affected both conditions equally, since it was still possible to observe a significant genotoxic effect by the I group in every parameter, making the

observable genotoxicity valid. Curiously, purines showed to be less affected with this problem, since the a.u. are lower in FPG-specific damage graph than in the other parameters.

When it comes to penoxsulam genotoxic effects, it was only observed in the non-specific damage parameter (figure 7). Moreover, and since no oxidative damage was signalized by FPG and EndoIII enzymes (figure 8 and 9), penoxsulam appeared to not be able to induce oxidative DNA damage. In a study of Patetsini *et al.* (2013), a lower concentration of penoxsulam (0.05 µg L^{-1}) showed to be genotoxic (assessed by the comet assay) after 7 days, in a marine mussel (*Mytilus galloprovincialis*). This study also stated that pesticides cause significant changes in the immune response of the tested organism, subsequently leading to the release of ROS. Therefore, the incapability of DNA lesion-repair enzymes to detect oxidative damage may be related to the exposure time (2h), which may have been insufficient to induce this kind of damage. Adding to these facts, Costa *et al.* (2018) demonstrated that male crayfish, exposed to a similar concentration of penoxsulam (20 µg L^{-1}), for 7 days, had neither significant non-specific nor oxidative DNA damage.

When analyzing the DNA damage classes concerning the exposure to penoxsulam, it is possible to observe a predominant class in the NC group (class 1) in every parameter, while for the P group, in varies between class 1 in non-specific damage (table III), class 1 and 2 in FPG-associated damage (table IV), and class 2 in EndoIII-associated damage (table V). Nevertheless, the sub-total values demonstrate a clear difference between the NC and P groups, particularly in the EndoIII-associated damage table. Curiously, the same parameter did not show a significant different between the NC group and P group. This peculiarity may be observed due to the high number of nucleoids of class 1 in the penoxsulam group, that is not accounted for in the sub-total value, leading to this difference. Consequently, it also points to the lack of pyrimidines oxidative damage.

In summary, every pesticide demonstrated to have a genotoxic effect, while environmentally realistic concentrations were tested, pointing also the possibility of the oxidative DNA damage occurrence, emphasizing the risk of pesticides to the environment.

4.2 Impact of Turex® in biological models

4.2.1 Effects in crayfish gill cells *ex vivo*

Previously, it was established the damaging effect of some chemically-based pesticides to the gill's cells of a non-target organism, at realistic concentrations. Therefore, efficient but not harmful alternatives to this type of pesticides are of great importance to be found, studied and applied. Biopesticides, based on naturally occurring substances, are considered nearly harmless to the environment and to non-target organisms, while still being efficient. Therefore, these

pesticides are commonly used as alternative to chemically-based pesticides. Thus, Turex®, a Bt-based insecticide, was selected to be tested, since it is considered as a good alternative to chemically-based insecticides. Due to the lack of knowledge concerning the effect of the biopesticide Turex® in non-target organisms, this study was designed as an attempt to understand the possible genotoxic effects to gill cells of the crayfish *Procambarus clarkii*, using the *ex vivo* approach presented above. Since there are no references in the literature concerning Turex® or *Bacillus thuringiensis* concentrations in ecosystems, a wide range of five concentrations was chosen, based on the usual scale of environmental concentrations of other pesticides and based on concentrations chosen in unpublished data of the work group concerning this biopesticide. Considering these, none have demonstrated to be genotoxic to crayfish (figure 10). This complies with what has been reported and observed about this biopesticide, since several studies (Sanchis 2011, Czaja *et al.* 2015, Boisvert and Boisvert 2000) have stated that only the target insects have the specific receptors and pH for the biopesticide to be effective, making it safe for non-target organisms. Boisvert and Boisvert (2000) performed a review with a compilation of results of several experiments, carried out in different classes of organisms, where the effect of Bt was evaluated. In their work, the tested organism most closely related to *Procambarus Clarkii* was another crayfish, *Orconectes limosus*, and it concluded that at higher concentrations, Bt did not affect this organism (Becker and Margalit 1993). Even observing the results for the organisms of the Decapoda order (to which *P. Clarkii* belongs to) no effects were observed in any organism, using either recommended dosages (according to the labels or producers) or overdosing (5 to 1000 times the recommended dosage) (Garcia *et al.* 1980, Merritt *et al.* 1989, Brown *et al.* 1999, Roberts 1995).

When comparing Turex® with the previously tested chemically-based insecticide dimethoate, it was possibly to infer, based on the results obtained in this study, that Turex® may be considered as less harmful to crayfish gill cells, posing thus a lower environmental risk when compared to dimethoate. However, the lack of knowledge concerning the realistic environmental concentrations must be considered.

4.2.2 Effects in liver cell line *in vitro*

Studies about the possibility of Turex® and/or its components having impact on humans, for example in occupational workers and consumers, have been scarcely performed, highlighting the importance of perform additional studies. The Panel on Plant Protection Products of the Norwegian Scientific Committee for Food Safety (VKM 2016) stated that the intake of Bt spores as residues is possible, specially by the intake of sprayed food items. These spores, when stored under certain conditions, can cause intestinal illness, resulting from the production of enterotoxins by vegetative Bt cells. Considering that Turex®, Bt cells or its insecticidal components may reach different tissues of the human body, the study of Turex® effects on HepG2 cell line appeared to

be pertinent. In resemblance to what was determined in the topic above, and since no guidelines concerning Bt-based pesticides concentrations in humans have been established, a wide range of five different concentrations was chosen. These were based on previous attempts with lower concentrations that induced no response, and to have a more inclusive approach of possibly different concentrations levels present in the environment.

When no previous activation was performed, only the highest concentration, 2000 µg L^{-1}, was able to be cytotoxic, but only after 48 hours (figure 11A). When analysing previous studies that evaluated the effect of Bt proteins in cell lines, the choice to use activated non-insecticidal Bt was generalised in most studies, therefore comparisons with the results obtained here were quite difficult. In previous studies (Kim *et al.* 2000, Yamashit *et al.* 2000, Yamashita *et al.* 2005) toxins from non-insecticidal *B. thuringiensis* demonstrated strong cytocidal activities against cell lines, including HepG2 (Yamashita *et al.* 2005), in experiments up to 24 hours, where some Bt proteins had structural and functional similarities to insecticidal Cry proteins. These studies also determined that different proteins may affect both cancer and healthy cell lines, and that the protein activity was usually higher when previous activation was performed. Nevertheless, the study of Yamashita *et al.* (2005) demonstrated that the tested concentration of specific Bt proteins, at concentrations up to five times higher than the highest concentration of Turex® used in the present study, were able to induce a cytotoxic effect in HepG2 cell line, reaching up to near 100% mortality after 24 hours. Another study (Kim *et al.* 2000) performed a dose-response curve of an inclusion protein of an isolate of Bt, in which, concentrations similar to those that was used of Turex® demonstrated to be cytotoxic to MOLT-4 cell line and normal T cells. Even though these studies are focused in non-insecticidal Bt proteins, there is study where only higher concentrations (around and above 1000 µg L^{-1}) can affect cell lines, as observed in the present study.

Similarly to what has been described in different studies (Kim *et al.* 2000, Thomas and Ellar 1983, Yamashita *et al.* 2000, Yamashita *et al.* 2005), an additional treatment was performed, where Turex® was activated with NaOH to mimic the biopesticide that, for some type exposure or process, could have passed through a basic pH treatment, ending up in activating and solubilizing the insecticidal protein, leading to a better understanding of the possible effects of Turex® in different conditions. When it came to the effects of pre-activated Turex®, cell viability was not negatively affected by the NaOH controls (figure 11B), while activated Turex® induced a cytotoxic effect with the highest concentration (T5), after 24 hours, and with the three higher concentrations (T3, T4 and T5), after 48 hours (figure 11C). This fact is corroborated with some of the studies referenced above, and it also demonstrated that this basic pH process appeared to be effective in activating some or even all the present proteins (Cry1Ac, Cry1C, Cry1D and Cry2A) (VKM 2016), that ended up being cytotoxic. Moreover, it was also observed that cytotoxicity presented a concentration-dependence.

After the assessment of Turex® cytotoxicity, its genotoxic potential was also determined. In what concerns to the model genotoxicant (hydrogen peroxide - PC), it had the expected high genotoxic effect, as stated by different studies (Benhusein *et al.* 2010, Nousis *et al.* 2005).

Studies concerning the possible genotoxic effect of Bt-based biopesticides are nearly inexistent. Despite this, a study aimed to compare a Bt-based pesticide with a deltamethrin-based emulsifiable concentrate, demonstrated that the tested biopesticide, Protecto®, did not induce DNA damage to rats that were daily exposed for 4 weeks, unlike the chemically-based pesticide that was considered as toxic (Ismail and Mohamed 2012). No further tests relating Bt and DNA damage were found until the present date.

In what concerns to the non-activated Turex®, the three lower concentrations (T1, T2 and T3) showed to have a genotoxic effect, after 48 hours (figure 12A), while the activated biopesticide was not able to induce DNA damage, neither after 24 nor 48 hours (figure 12B). These facts may be due to several hypothesis such as the non-activated Turex® ability to enter the cell membrane or even due to the activation of some protective mechanism when gill cells encountered activated biopesticide, maintaining it out of the cell. On the other hand, it may be due to the possible similarity between cytoplasmatic enzymes and the insect gut proteases that activated the crystal toxins, which may lead to the posterior activation of Turex® once inside the cell. However, it was observed that only the lower concentrations of non-activated Turex® were able to induce DNA damage, which may be explained by a possible contaminant limit for the cell, that when reached, could possibly induce a protective mechanism of the cell, limiting DNA damage or even the entrance of the contaminant.

5. Conclusions

This work allowed to validate the *ex vivo* approach with gill cells of the crayfish *Procambarus clarkii*. The exposure length of 8 hours was excluded due to its cytotoxic effect, while the 4-hour length was excluded due to its decreased DNA integrity. Therefore the 2 hours exposure showed to be the most suitable exposure time.

Moreover, this approach was successfully applied to assess the genotoxicity of three different pesticides by using the comet assay, in a fast and reliable way. The genotoxic effect of dimethoate, imazalil and penoxsulam, at environmental realistic concentrations, was demonstrated. Oxidative DNA damage was not detected by the net enzyme-sensitive sites (NSS) but should not be excluded for dimethoate and imazalil.

When it came to the Bt-based biopesticide, Turex®, it did not affect the DNA integrity of the crayfish gill cells, using the *ex vivo* approach addressed above, while when HepG2 cell line was

exposed *in vitro*, it demonstrated to be affected. Cells viability was mainly affected when the biopesticide was activated, while DNA integrity loss was more evident with the non-activated biopesticide, both happening predominantly after 48 hours.

Finally, it was suggested that the use of chemically-based pesticides should be reconsidered (and minimized) as also substituted by potentially safer alternatives as the biopesticide Bt appears to be. Despite these considerations, further testing with Bt-based pesticides in different organisms and human cell lines should be performed.

6. Future prospects

In order to better evaluate and predict the possible negative effects of pesticides in the ecosystem, it is necessary to develop new ways to apply pre-existing methodologies or even to create new methodologies. In this context, the *ex vivo* approach applied to crayfish gill cells, and using the comet assay, demonstrated to be a useful tool accessing waterborne contaminates' effects, in a fast and reliable way. Also, this approach allowed the reduction of the number of organisms, when compared to similar *in vivo* experiences, from 42 organisms in *ex vivo*, to 214 organisms in a corresponding *in vivo* experiment. Thus, it would be of great interest to continue to improve this approach, testing also different mediums to increase the cells' lifespan, while maintaining a non-culture format. Studying different tissues and organisms would also be a valuable add-on, since it could increase the range of classes of organisms and environments that could be tested, enhancing the utilization of the *ex vivo* approach. Adding to this, an *in vivo* experiment corresponding to the present *ex vivo* experiment, would be of value for the comparison between results, leading to a better comprehension of a possible data extrapolation and relation between approaches.

As demonstrated here, pesticides of different chemical classes, at environmental realistic concentrations, can have a great impact in non-target organisms, such as crayfish, by affecting DNA integrity of gill cells. This tissue is of high importance due to its respiratory function, which in turn may affect the organism at higher levels, such as the survival of the organism. In order to decrease the risk of such hazardous effects, imposed by the application of pesticides, from happening in different organisms, some measures must be taken. For example, an effort to decrease the used quantities of pesticides should be a priority, while adopting new strategies, as well as, to choose safer and more ecologic alternatives to pesticides.

Biopesticides have been a widely used alternative to chemically-based pesticides, generally considered a safe and ecologic choice. However, it was observed that the alleged safety of a

Bacillus thuringiensis based biopesticide is not that linear, demonstrated by the negative consequences observed in the liver cell line.

On the other hand, and as stated previously, humans may be affected by this biopesticide. Therefore, the evaluation of the cytotoxic and genotoxic effects of Turex® in a cell line, such as HepG2, appear to be of great interest by helping to fulfil the knowledge gap concerning this pesticide effects. Turex® negative consequences in HepG2 cell line may illustrate the possible consequences for humans, that might end up containing Bt compounds in the circulatory system, either by ingestion, contact or the respiratory system.

Bearing this in mind, biopesticides should be further investigated when it comes to their persistence in the environment and concerning their negative effects on non-target organisms. Finally, it will be extremely interesting to evaluate the presence and accumulation of this biopesticides in the human body, as well as the possible consequences, instead of considering an alleged safety.

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