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Caracterização ecotoxicológica e formulações alternativas ambientalmente mais favoráveis de um herbicida comercial (Winner Top®)

Ecotoxicological characterisation and ecofriendlier alternative formulations of a commercial herbicide (Winner Top®)

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Toxicologia e Ecotoxicologia, realizada sob a orientação científica da Doutora Joana Luísa Pereira e da Doutora Tânia da Silva Vidal, Investigadoras em Pós-Doutoramento, e do Professor Doutor Fernando Gonçalves, Professor Associado com Agregação, todos do Departamento de Biologia da Universidade de Aveiro e do Centro de Estudos do Ambiente e do Mar.

Aos meus pais, irmã e avós

“In every conceivable manner, the family is link to our past, bridge to our future.”

Alex Haley

o júri

presidente

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agradecimentos

E chega assim ao fim esta importante etapa da minha vida académica, que só foi possível concretizar com o apoio de pessoas especiais, às quais quero agradecer.

Ao Professor Doutor Fernando Gonçalves por me ter acolhido no seu grupo de investigação para o desenvolvimento da dissertação e pela preocupação que sempre demonstrou em proporcionar as melhores condições para o desenvolvimento de todo o trabalho.

À minha orientadora Doutora Joana Pereira não só pelo seu profissionalismo, mas também por ser uma pessoa muito acessível, preocupada e amiga. Obrigada por toda a sabedoria, conselhos, dedicação e pelas explicações estatísticas e revisão da dissertação.
À minha co-orientadora Doutora Tânia Vidal pela dedicação, disponibilidade, positivismo e pela preciosa ajuda laboratorial, que foi muito importante em momentos de maior aperto.
Quero agradecer às duas pela confiança no meu trabalho e por me terem dado sempre muita liberdade para trabalhar e por vezes errar, porque é com os erros que se aprende e se ganha experiência. Aprendi muito com as duas.

Ao Professor Doutor António Nogueira pela disponibilidade, boas ideias e domínio dos modelos de toxicidade de misturas de químicos, que contribuíram para um aumento da qualidade do trabalho.

Ao Engenheiro João Faria, diretor de marketing da empresa Selectis, por ter atenciosamente atendido ao meu pedido e pela intervenção que teve na empresa para que me fossem facultadas amostras do herbicida Winner Top®.

Aos colegas do grupo LEADER pela boa disposição, incentivo, bom ambiente de trabalho e por estarem sempre disponíveis para ajudar.

Aos meus amigos, pelo apoio, desabafos, motivação, conselhos e pelos momentos de descontração, que também são fundamentais para que as coisas não descarrilem. Obrigada Marisa, Cátia F., Sofia, Cátia P., Joana, Inês, Vera e Maryse. Obrigada ao meu namorado e amigo, Eduardo, por toda a paciência e apoio incondicional.

E de grande importância, à minha família, que me deu sempre muita liberdade para tomar as minhas próprias decisões, pela confiança e por sempre acreditarem em mim. Obrigada mana pelas dicas e correções no inglês.

palavras-chave

Produtos fitofarmacêuticos, toxicologia aquática, organismos não-alvo, *Lemna minor*, *Lemna gibba*, *Raphidocelis subcapitata*, *Chlorella vulgaris*, *Portulaca oleracea,* toxicidade de misturas, Winner Top®, nicosulfurão, terbutilazina, formulantes

resumo

Tem-se assistido a um uso potencialmente abusivo de produtos fitofarmacêuticos, com consequentes efeitos ambientais. Assim, o desenvolvimento de produtos mais eficazes e amigos do ambiente é um dos grandes desafios da atualidade. Neste contexto, este trabalho teve como principais objetivos: (i) avaliar a toxicidade dos formulantes ou adjuvantes utilizados nas formulações dos produtos comerciais, utilizando um herbicida modelo (Winner Top®), de forma a verificar se a designação destes ingredientes como "inertes" é realmente cabível; (ii) desenvolver uma nova metodologia para a formulação dos produtos comerciais visando a manipulação do rácio dos seus constituintes, mantendo a eficácia contra as espécies alvo e exercendo, ao mesmo tempo, menor toxicidade sobre organismos não-alvo.

Os ingredientes ativos do Winner Top® (nicosulfurão e terbutilazina) foram testados individualmente e em mistura, considerando o rácio usado na formulação comercial e rácios alternativos. A formulação comercial foi também testada para análise da contribuição dos formulantes para a toxicidade do herbicida. Duas espécies de algas (*Raphidocelis subcapitata* e *Chlorella vulgaris*) e duas espécies de macrófitas (*Lemna minor* and *Lemna gibba*) foram selecionadas como organismos não-alvo para estes testes, que avaliaram os efeitos dos tóxicos no seu crescimento. Foi também realizado um teste de vigor vegetativo com um organismo alvo, a beldroega (*Portulaca oleracea),* para se testar a eficácia de formulações alternativas à do composto comercial. Estas formulações foram estabelecidas tendo em conta as concentrações de cada ingrediente que não exerciam efeitos intoleráveis em *Lemna minor*.

Os testes de toxicidade individual revelaram que a terbutilazina foi o principal inibidor de crescimento para as microalgas e o nicosulfurão para as macrófitas. Por outro lado, a mistura dos ingredientes ativos no mesmo rácio da formulação comercial foi aparentemente mais tóxica do que a formulação comercial. Logo, os formulantes do Winner Top® não serão inertes. Por outro lado, o teste de toxicidade de misturas sinalizou que a combinação dos ingredientes ativos tem

uma ação antagonista, dependente do nível de efeito, na inibição do crescimento do organismo não-alvo. Estas evidências reforçam as recomendações que têm vindo a ser feitas acerca da necessidade de considerar as formulações, e não os seus componentes isoladamente, na análise de risco prévia à autorização de comercialização de pesticidas.

A eficácia, contra a espécie alvo, de formulações alternativas seleccionadas foi equivalente ou em alguns casos superior à da formulação usada no composto comercial, tendo-se verificado que um dos ingredientes ativos não adiciona potencial letal relevante à formulação. Estes resultados permitem sugerir que a manipulação racional do rácio entre os constituintes das formulações comerciais, tendo por base os efeitos ambientais esperados, pode ser uma alternativa para as indústrias de agroquímicos que pretendam desenvolver formulações mais amigas do ambiente. É importante notar ainda que, considerando o exemplo estudado, esta modificação do *modus operandi* no desenvolvimento das formulações não implicaria perda de eficácia do produto final.

keywords

Plant protection products, aquatic toxicology, non-target organisms, *Lemna minor*, *Lemna gibba*, *Raphidocelis subcapitata*, *Chlorella vulgaris*, *Portulaca oleracea,* mixture toxicity, Winner Top®, nicosulfuron, terbuthylazine, formulants

abstract

A potentially abusive use of plant protection products with consequent environmental effects has been reported. Thus, the development of more efficient and environmentally friendlier products is a major challenge nowadays. In this context, the main objectives of this study were: (i) to evaluate the toxicity of formulants or adjuvants used in the commercial products using a model herbicide (Winner Top®), in order to verify whether they are as inert as they are supposed to be; (ii) to develop a new methodology to rule the formulation of commercial products focused at the manipulation of the ratio between its constituents that can maintain the efficacy against the target pests but having reduced environmental toxicity.

Winner Top®'s active ingredients (nicosulfuron and terbuthylazine) were tested singly and in mixture, considering the ratio used in the commercial formulation and alternative ratios. The commercial formulation was also tested to assess the contribution of formulants to the overall toxicity of the herbicide. Two microalgae (*Raphidocelis subcapitata* and *Chlorella vulgaris*) and two macrophytes (*Lemna minor* and *Lemna gibba*) were used as non-target organisms in these tests intending to evaluate growth inhibition. A vegetative vigour test was also performed with a target organism, the purslane (*Portulaca oleracea),* in order to test the efficacy of alternative formulations to that used in the commercial product. These formulations were established taking into account the concentrations of each ingredient that did not have intolerable effects on *Lemna minor*.

Single chemical tests revealed that terbuthylazine was the strongest microalgae growth inhibitor and nicosulfuron was the strongest macrophyte growth inhibitor. On the other hand, the mixture of the a.i.s at the formulation ratio was apparently more toxic than the commercial formulation, thus Winner Top® formulants are not inert. On the other hand, mixture toxicity tests indicated that the combination of the active ingredients has a effect-level dependent antagonistic action in

inhibiting the growth of non-target organisms. These evidences reinforce the need to consider the formulations, rather than only their isolated components, in risk assessment prior to the authorization for pesticides marketing.

The efficacy against the target species of tested alternative formulations was equivalent or higher than that of the commercial formulation. Moreover, one of the active ingredients does not add any relevant lethal potential to the formulation. These results suggest that the rational manipulation of the ratio between formulation components, based on expected environmental effects, may be an alternative for agrochemical industries that aim to develop environmentally friendly formulations. Considering the present example, it is also noteworthy that this modification of the *modus operandi* in the development of pesticide formulations does not necessarily imply losses in efficacy.

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CHAPTER 1 - General introduction and objectives

1.1. Introductory note

Plant protection products (PPPs) are extensively used in agriculture to keep agricultural production rates (Carlile, 2006). However, their massive use has resulted in negative effects on human health and the environment (Bjørling-Poulsen et al., 2008; Carlile, 2006; Cox and Surgan, 2006; Hossard et al., 2014; Mnif et al., 2011).

PPPs are formulations composed by one or more active ingredients (a.i.s) plus other chemicals also known as inert ingredients. The so-called inert ingredients are also designated as 'formulants'/'adjuvants' (Cox and Surgan, 2006; Mesnage et al., 2014; Surgan et al., 2010). An up-to-date controversy is the role of these ingredients in the environmental toxicity of PPPs. Only the a.i.s and formulants or adjuvants proven to represent an environmental hazard *per se* are disclosed (EC, 2009), i.e. the interaction between formulation components is disregarded. Ironically, the formulants or adjuvants are added to the PPP recipe because they can interact with other components constituting the PPP mixture to improve its overall stability, delivery and efficacy against the target pest (Castro et al., 2014; Surgan et al., 2010). Then, it is reasonable to hypothesize that such interactions may also play a role in the toxicity of the PPP to non-target environmental receptors. This overall controversy has led to the first questions ruling this work: are formulants/adjuvants truly inert to non-target organisms?

As a follow-up to a somewhat expected negative answer to this first question, a consequent challenge was set towards finding alternatives to the development of environmentally friendlier agrochemical formulations. The agrochemical industry has already been forced to innovate in the formulation of its products in order to replace substances lost due to the new registration requirements. Common strategies followed in this context have been the replacement of formulants or adjuvants by natural products (greener equivalents) or/and the improvement of PPP delivery techniques (Cantrell et al. 2012; Dayan et al. 2009; Singh et al. 2013). An alternative approach proposed in this study is to manipulate the components ratio within the commercial formulation. This approach would be based on deviations from the reference models of mixture toxicity, which denote synergism, antagonism, dose-level and dose-ratio effects. The main expectation here was the spotting of combinations with antagonistic behaviour towards non-target environmental indicators that would represent eco-friendlier formulations as compared to the commercial product.

The so-called inert ingredients are added to PPP formulations to increase their effectiveness against target species, which are often nuisances affecting agricultural production. However, these substances can easily end up in surrounding aquatic ecosystems. In this way, in order to answer to the first question (are formulants truly inert to non-target organisms?) two species of algae (*Raphidocelis subcapitata* and *Chlorella vulgaris*) and two species of macrophytes (*Lemna minor* and *Lemna gibba*) were selected as models in this study, representing aquatic non-target organisms. The PPP selected as model was the commercial herbicide Winner Top®, a 2-way formulation using terbuthylazine and nicosulfuron as active ingredients. The so-called inert ingredients are not individually identified on the product label, with the formulation being classified as an

oil dispersion (Selectis, 2012). As a first stage, the so-called inert ingredients role in the environmental toxicity of Winner Top® was studied based on several tests with the selected non-target organisms for a general characterization of the aquatic toxicity of the commercial formulation Winner Top®. The contribution of formulants to the overall toxicity of the product was specifically focused. The toxicity of each of the two a.i., that of the commercial formulation Winner Top® and that of a customized mixture of the a.i. respecting their ratio in the commercial formulation were compared for the purposes. In a second stage, alternative formulations of the two active ingredients used in Winner Top® were tested for efficacy against a target weed, *Portulaca oleracea* (an herbaceous terrestrial plant) and their environmental friendliness was confirmed using a non-target model, *Lemna minor* (an aquatic plant that was also used in the first part of the present work).

1.2. Pesticides, their use worldwide, and their environmental effects

1.2.1. Definition and classification of pesticides

Pesticides can be defined as chemicals widely used in agriculture to maintain and increase crop yields by reducing weeds, pests and diseases (Bjørling-Poulsen et al., 2008; Carlile, 2006; Castro et al., 2014; Hildebrandt et al., 2007; Katagi, 2008; Pereira et al., 2009). However, these chemicals also have other applications such as in industry, trade (e.g. restaurants), public spaces (e.g. gardens) and even in our homes to prevent and combat the attack of rodents, insects, fungi, etc. (Bjørling-Poulsen et al., 2008; Carlile, 2006).

Pesticides can be classified according to the organism they target, e.g. insecticides targeting insects, fungicides for fungi, herbicides for plants, molluscicides for molluscs, rodenticides for rodents (Carlile, 2006; DGAV, 2015a; Marrs and Ballantyne, 2004). They can also be classified according to chemical structure (chemical class), mode of action (e.g. anticholinesterasics, growth inhibitors) or intake route (e.g. systemic vs contact). Some classifications are a blend of the above, with the target organism being generally used as the major division and the chemical class as a subdivision (Carlile, 2006; Marrs and Ballantyne, 2004).

Fungicides, herbicides and insecticides are the three major groups of pesticides consumed nowadays (see sections 1.2.2.1 and 1.2.2.3 below). Fungicides have multiple applications such as controlling plant pathogens (fungal microorganisms) in agriculture (Carlile, 2006; DGAV, 2015a; Marrs and Ballantyne, 2004), protect the timber from the attack of wood-rotting fungi, prevent or cure fungal infections on humans and other organisms (Carlile, 2006). Fungicides can be classified according to their chemical class; for example: benzimidazole fungicides, carbamate and dithiocarbamate fungicides, inorganic fungicides, etc. (EC-European Communities, 2007; Pscheidt, 2016). Herbicides are used to kill unwanted plants in agriculture (DGAV, 2015a; Vidal et al., 2011) and in urban areas at a smaller scale (Joly et al., 2013). They can be classified according to their chemical class or mode of action. Common classification combinations are e.g. acetyl CoA carboxylase (ACCase) inhibitors, ALS (acetolactate synthase)/AHAS (acetohydroxy acid synthase) inhibitors, microtubule assembly inhibitors (Alberta, 2015). Finally, insecticides are used in agriculture to control structural pests of insects that can cause problems to crops (DGAV,

2015a) and they are also heavily used in urban areas by homeowners and professionals for mosquito or structural pest control and landscape treatment (Overmyer et al., 2003; Templeton et al., 1998). Insecticides and acaricides are frequently represented together i.e. as belonging to the same group. Similarly, acaricides are used to combat structural pests of mites (DGAV, 2015a). Based on their chemical class/properties, insecticides and acaricides are generally divided in: carbamates, organochlorines, organophosphates, pyrethroids, biological products and other insecticides/acaricides (including insect growth regulators). It is not uncommon that the same pesticide product can exert both insecticidal and acaricidal effects (DGAV, 2015a).

1.2.2. Pesticides use or abuse

Nowadays, agriculture plays a key role in the world economy since it provides food and raw materials needed to sustain a growing human population (Carlile, 2006; Fuentes et al., 2013), estimated to reach about 10 billion people in 2050 (Castro et al., 2014). For this reason, this sector relies on the use of various agrochemicals such as pesticides and fertilizers to ensure improvements in quality and yield of crops (Carlile, 2006; Fuentes et al., 2013; Knowles, 2008; Singh et al., 2013). The 1960s' Green Revolution in industrialized countries caused a significant increase in productivity by using these chemicals, mechanization and planting hybrid crops with higher yields (Hond et al., 2003; Mnif et al., 2011). From 1960 to 1990 the average yield of cereal crops increased more than 98% worldwide with the massive use of pesticides (Hossard et al., 2014), which figures the major relevance of agrochemicals nowadays.

On one hand, pesticides and other agrochemicals are of paramount importance as part of modern agricultural practices to sustain food supply, but on the other hand they represent a serious risk to human health and the environment through the impacts they drive in non-target organisms (Hond et al., 2003; Hossard et al., 2014; Surgan et al., 2010; Vidal et al., 2011). In fact, the use of pesticides and other agrochemicals in agriculture constitutes a major source of diffuse pollution (Abrantes et al., 2009) threatening ecosystems in the surroundings. Many of the first generation pesticides like DDT persist in soil and aquatic sediments and bioconcentrate in organisms' tissues across different trophic levels (Mnif et al., 2011). Although these have been banned worldwide and despite the effort to develop new alternatives with lower environmental impacts, the contamination picture is still worrisome.

High levels of pesticide residues are found in various environmental matrices (Konstantinou et al., 2006; Planas et al., 1997) and these have been proving able to negatively affect water quality and biodiversity (Abrantes et al., 2009; Beketov et al., 2013; Hossard et al., 2014). Also, the amounts of pesticide residues found in food and drinking water have been raising (Damalas and Eleftherohorinos, 2011). Approximately 300 different types of pesticides residues have been reported in food products of European origin and most of them are not included in mandatory monitoring programs (Bjørling-Poulsen et al., 2008), which underestimates the actual contamination scenario.

In order to have a more concrete idea about man's dependence on these compounds some statistics regarding its use are presented below. A note is worth making on the standard designation Plant Protection Products (PPPs), which is given by EU to group

pesticides (insecticides, herbicides, fungicides, among others) and other agrochemicals with no pesticide activity like plant growth regulators (DGAV, 2015a; EC-European Communities, 2007). This designation will be used occasionally throughout this work. Also, the standard acronym AS, for active substance, is used interchangeably in this document with a.i., for active ingredient, which is a more common designation in scientific studies.

1.2.2.1. PPPs use worldwide

According to the OECD (2008) report about the environmental performance of agriculture in OECD countries[1], the overall pesticide use (sales) declined by 5% over the period between 1900-1992 (867 588 tonnes of AS) and 2001-2003 (820 826 tonnes of AS). However, this declining was not common to all OECD countries. Concerning the largest users (representing 75% of total OECD pesticide use in 2001-2003), pesticides use increased in Italy, Mexico and Spain and decreased in France, Japan and United States. In other countries like Greece, Mexico, Poland, Portugal and Turkey, the use of these compounds increased by 20% in the same period. In Portugal, this growth (from 13 200 tonnes of AS in 1900-1992 to 16 661 tonnes of AS in 2001-2003) was mainly due to the rise of the horticultural sector. In opposite, in other countries like Austria, Czech Republic, Denmark, Hungary, Japan, Netherlands, Norway and Switzerland, pesticide use decreased over 20% due to a combination of factors such as a decline in crop production, the use of incentives and taxes, the adoption of integrated pest management practices or the expansion of organic farming.

In some cases, pesticide use benchmarks obtained based on sales (in tonnes of AS) are not enough clear in explaining the changes that have occurred over the last decades. In fact, sales decrease can also be explained by the development of new pesticide products with similar levels of high efficacy achieved by lower dosages (*e.g.* pyrethroid fungicides). These products can be widely used but their small quantities have a reduced contribution to the overall mass of sales (EC-European Communities, 2007).

Worldwide, including non-OECD members, the pesticide use was about 2.29 billion kg of AS in 2001 (Kiely et al., 2004) and more recent data show that this value increased to about 2.36 billion Kg of AS per year in 2007 (Grube et al., 2011).

[1] OECD countries in this accounting period - Australia, Austria, Belgium, Canada, the Czech Republic, Denmark, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Japan, Korea, Luxembourg, Mexico, the Netherlands, New Zealand, Norway, Poland, Portugal, the Slovak Republic, Spain, Sweden, Switzerland, Turkey, the United Kingdom and the United States.

1.2.2.2. PPPs use in the European Union (EU)

According to the Eurostat (EC-European Communities, 2007), the total amount of PPPs used in the EU ranged between 200 000 and 250 000 tonnes of AS per year in the period between 1992 and 2003. In 2003, the benchmark was around 220 000 tonnes of AS (EU-25 Member States[2]). In this year, fungicides represented the most consumed category (49%), followed by herbicides (38%) (Figure 1).

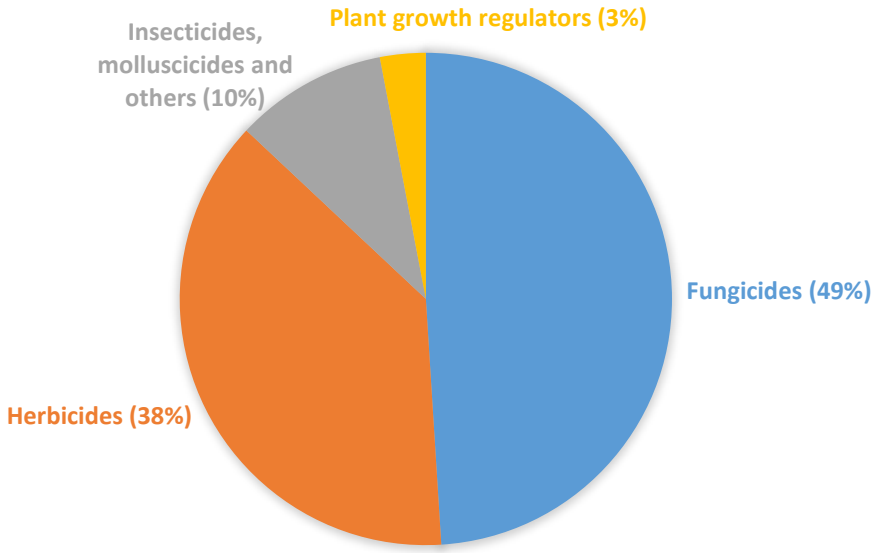


Figure 1 – Distribution of PPPs use by category in the EU-25, given as % total quantity of AS in 2003 (adapted from EC-European Communities, 2007).

The Top 5 Member States with the greatest use of PPPs (nearly 75% of the total) was comprised by France (28%), Spain and Italy (14% each), Germany (11%) and United Kingdom (7%) (Figure 2). Portugal used 6.1% of the total amount considered (13 321 tonnes of AS).

[2] EU-25 countries - Austria, Belgium, Cyprus, Denmark, Finland, France, Germany, Greece, Ireland, Italy, Luxembourg, Malta, Netherlands, Portugal, Spain, Sweden, United Kingdom, Czech Republic, Estonia, Hungary, Latvia, Lithuania, Poland, Slovenia and Slovak Republic.

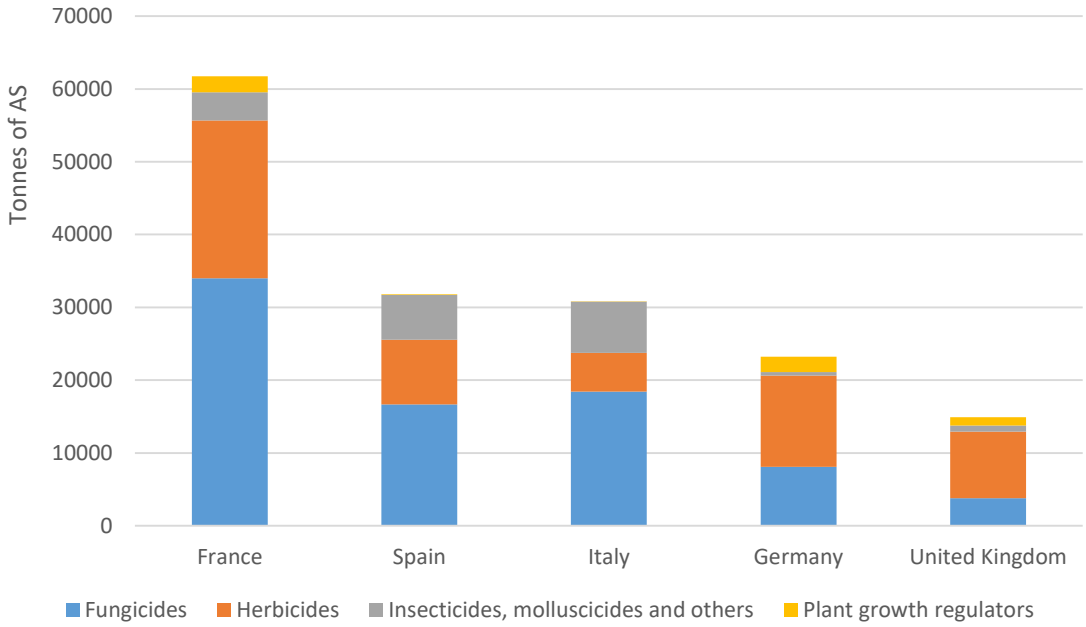


Figure 2 – Quantity of PPPs used by the Top 5 EU Members States in tonnes of AS for EU-25 in 2003 (adapted from EC-European Communities, 2007).

Updates to the information above have been made by the Eurostat, but not consistently and covering all EU Member States. The data available on PPP sales for the EU-28[3] countries until 2014 indicate the consumption of these compounds increased to close to 400 000 tonnes of AS per year; sales are apparently dominated by the same member states as in 2003, added Poland with even a higher record than the United Kingdom (Eurostat, 2016a, 2016b). Through a search by type of PPPs sold for each EU country, fungicides were confirmed as the most sold PPP class in 2014, followed by herbicides (Eurostat, 2016b, 2016c).

1.2.2.3. PPPs use in Portugal

According to OECD (2008), Portugal bucked the global trend over the period 1900-1992 to 2001-2003 by increasing the amount of pesticides used through time. In 2001, when first sales data began to emerge, 9355 tonnes of PPPs were sold, and in 2002, 17 037 tonnes of AS were sold (Vieira, 2012). According to EC (2007), Portugal used 13 321 tonnes of PPPs AS in 2003, the market sales being largely dominated by fungicides (about 11 000 tonnes of AS), with herbicides standing at the 2nd place (about 1700 tonnes of AS). In this same year, 84% of all PPPs were used in vineyards and the most important AS applied on vines was sulphur, which is used in several formulations of fungicides and insecticides (DGAV, 2015b).

[3] EU-25 countries plus Bulgaria, Romania and Croatia.

Between 2008 and 2010, total sales followed a declining trend but herbicide sales increased (DGADR, 2011). In 2010, the total amount of PPPs sold was around 13 795 tonnes of AS, 1% less than in 2009. Fungicides kept the highest sale records (69% of total sales), similarly to 2003, but herbicide sales increased by 20% compared to 2009 (15% of total sales) as deducible from the interpretation of Table 1. Glyphosate, terbuthylazine and MCPA were the AS most sold, with glyphosate and amitrole being the major drivers of the mentioned increase in herbicide sales (DGADR, 2011).

Table 1 - Herbicide sales in 2010 by AS and the respective comparison to 2009 (DGADR, 2011). The two AS used in the present study were highlighted bold.

AS	Quantitative sold (Kg)	Comparison to 2009 (Kg)
Glyphosate	1427650	+395983
Terbuthylazine	186512	+4959
MCPA	33474	-19759
Amitrole	29049	+13536
Oxyfluorfen	27580	-3872
Metribuzin	16070	+3863
2,4-D	15338	-3222
Triclopyr	12485	-9093
Diflufenican	7499	-2302
Propanil	6000	-10195
Nicosulfuron	5905	+1878
Chlortoluron	4217	-3919
Bromoxynil	2870	-704
Diclofop-methyl	1958	-5578
Metamitron	161	-308
Isoproturon	90	-60
Trifularin	0	-1923

Moreover, and now specifically considering the pesticides used in the present study, terbuthylazine and nicosulfuron represent a significant part of sales with an increasing trend recognised from 2009 to 2010 (Table 1).

The sales of PPPs in the country declined between 2011 and 2013 but then increased in 2014 (Figure 3). In 2013, 10 100 tons of PPPs were sold, which was 18.8% less than in 2012. In the period between 2011 and 2013, 3900 less tonnes of these products were sold (INE, 2015, 2013). Fungicides remained the most important group (71.1% of total sales in 2013; Figure 3). However, it is important to refer that sulphur represented 68.1% of the total of fungicide sales in 2013 (71.4% in 2012). Herbicides followed a declining trend over these 3 years (1996 tonnes of AS in 2011, 1769 tonnes of AS in 2012 and 1611 tonnes of AS in 2013; INE, 2015). In 2014, 12 900 tons of PPPs were sold, inverting the downward trend. In this year, sulphur represented 69.7% of the total of fungicide sales. The use of herbicides increased to 2410 tonnes of AS (INE, 2016).

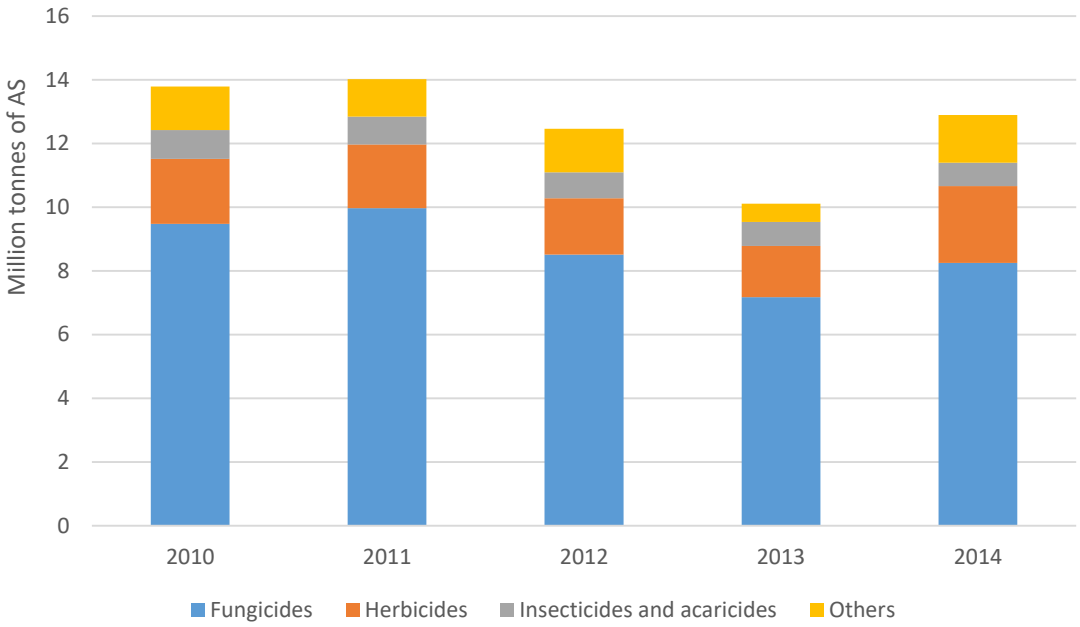


Figure 3 - PPP sales by category in Portugal, given in million tonnes (adapted from INE, 2016, 2015, 2014).

1.2.3. Environmental contamination

Pesticides are one of the few toxic substances that are intentionally applied in the environment (Gavrilescu, 2005). Their persistent and wide use evidences that pesticides are indispensable to sustain agricultural production, but the other side of the coin is a continuous inflow of their residues into different environmental matrices (e.g. water, soil) causing frequently hazardous contamination scenarios (Abrantes et al., 2010; Battaglin et al., 2014; Carriger and Rand, 2008; Cerejeira et al., 2003; Konstantinou et al., 2006). There have been identified pesticide residues almost all over the globe, including polar regions (Carlile, 2006). It is further worth noting in this context that only a small portion of the total amount of pesticides used is estimated to reach the intended sites of action because the larger proportion is lost via e.g. spray drift, off-target deposition, run-off, photodegradation (Damalas and Eleftherohorinos, 2011; Gavrilescu, 2005; Perre et al., 2015). As a final outcome, there are several examples in the literature linking exposure to PPP residues to a significant ecosystem risk (e.g. Abrantes et al., 2010, 2009; Carriger and Rand, 2008).

Agricultural soil acts as the primary recipient for pesticides (Gavrilescu, 2005; Hildebrandt et al., 2007; Pereira et al., 2009) since most techniques are based on direct or indirect application of these compounds into soil (Cerejeira et al. 2003; Gavrilescu, 2005). In this compartment, pesticides can be degraded, adsorbed onto organic matter of soil or lixiviate (Gavrilescu, 2005; Hildebrandt et al., 2007). The transport of pesticides within the soil depends on the chemical properties of soil particles, including their distribution and size, the amount of organic matter of the soil and even the type of pesticide and adjuvants involved. The fate of pesticides through soils also depends on application procedures and climatic conditions. The latter, plus soil characteristics have been recognized as the most important factors constraining the transport of these compounds in the environment (Damalas and Eleftherohorinos, 2011; Gavrilescu, 2005). Some of the most persistent

pesticides tend to lixiviate, acting as sources of groundwater contamination (Alva and Singh, 1991; Cerejeira et al., 2003; Gonçalves et al., 2007). This is concerning since groundwaters are used as irrigation and drinking water sources (Hildebrandt et al., 2007) and there are studies reporting concentrations of pesticides above the recommended safety levels for drinking water (Abrantes et al., 2010; OECD, 2008). The most water soluble and more persistent pesticides can indeed reach surface water bodies in significant amounts (Konstantinou et al., 2006; Wauchope, 1978).

The present study will focus its attention on surface water contamination by pesticides, which mostly follows transport through the soil matrix and occasionally through groundwater. It happens worldwide and it has been matter of concerns and debate within the scientific community (Albanis and Hela, 1998; Cerejeira et al., 2003; Huber et al., 2000; Konstantinou et al., 2006; Planas et al., 1997). Pesticides are moved from agricultural areas to surface waters through surface run-off and/or leaching. This transport pathway depends on soil characteristics, topography, agricultural practices and physicochemical properties of pesticides, namely vapour pressure, stability, solubility, pK_a, etc. (Damalas and Eleftherohorinos, 2011; Konstantinou et al., 2006; Larson et al., 1995). Significant advances have been made in the control and mitigation of point-source contamination of surface waters. In contrast, nonpoint-source (diffuse) contamination of surface waters has been found harder to control due to its intrinsic spatial, temporal and contextual variability. Pesticides and fertilizers indeed represent one of the main sources of diffuse surface water pollution (Carpenter et al., 1998; Loague et al., 1998), whose control has been experiencing very slow progress (Albanis and Hela, 1998; Konstantinou et al., 2006), thus representing a problem of increasing concern worldwide (Abrantes et al., 2009; Huber et al., 2000; Loague et al., 1998).

Following the recognition of this problem, regulatory agencies worldwide have been developing tighter screening protocols and authorization requirements, as well as comprehensive assessment instruments. Amongst these later, modelling tools have been developed that allow us to analyse the transport of pesticides from the point of application to the aquatic environment and estimate their final concentrations in surface water and groundwater. These modelling tools are integrated in risk assessment schemes required for the PPPs licensing process. They allow assessing expected levels of contamination in surface water and groundwater bodies, thus estimating putative threatening scenarios to the aquatic biota. The developed modelling tool that is currently used in the European Union is the FOCUS platform. It is an open-access software (downloadable from: http://esdac.jrc.ec.europa.eu/projects/focus-dg-sante). FOCUS was designed and developed within several European projects and it is used to calculate expected concentrations of PPPs in surface water and groundwater (PECs - Predicted Environmental Concentrations) depending on application rates and physicochemical properties of chemicals and european soils; it was developed after Regulation (EC) No. 1107/2009 (EC, 2009).

FOCUS is based on the application of mathematical models for the estimation of PECs. It is divided into several modules developed through a 4-level stepwise approach. The first step corresponds to a simple approach using simple kinetics and assuming a loading equivalent to a maximum annual application. The second step is similar to step 1 but accounts for a more realistic loading based on sequential application patterns. The third

step is based on a more detailed modelling approach, using realistic worst-case scenarios but taking into account agronomic and climatic conditions relevant to the crop considered and a selection of typical water bodies expected to be affected. The fourth step is similar to step 3 but also considers the range of possible uses related to cropping, soil, weather, field topography and aquatic bodies adjacent to fields (used to specific local situations). The software uses standard scenarios in order to uniform PEC assessment. In this context, ten realistic worst-case scenarios that collectively represent agricultural fields across Europe are used in step 3 to evaluate the contamination of surface waters by the use of a given active ingredient at the EU level. The fixed properties and characteristics defined for each of these scenarios are the mean annual temperature (°C), the annual rainfall (mm), the type of topsoil (Silty clay, sand, etc.), its organic carbon content (%), the slope involved (%) and the type of water bodies focused (stream, ditch…).

Three models are used by the FOCUS to estimate the fate of a substance in different environmental compartments after its application: MACRO, to estimate the contribution of drainage; PRZM, to estimate the contribution of runoff; and TOXSWA, to estimate the final PECs in surface waters. The estimated PECs for surface water bodies must typically be complemented with ecotoxicological data regarding aquatic organisms. A comparison between the estimated PEC and the lowest EC_{50}/LC_{50} retrieved in acute toxicity testing with aquatic organisms (algae, daphnia, fish…) is required throughout and for a definitive calculation of a Toxicity Exposure Ratio (TER; TER = EC_{50}/PEC). When chronic exposures are under assessment, the PEC calculated as the time-weighted average concentration over the appropriate time period is compared with the NOEC (No Observed Effect concentration) for the same or another aquatic organism. If the calculated TER is less than 10 which is the benchmark established in the Annex IV of the Regulation (EC) No. 1107/2009, it means that the use of the evaluated active substance has an unacceptable impact on the aquatic environment and further risk mitigation measures have to be considered (EC, 2009; EFSA, 2011, 2007; Linders et al., 2001). Relevantly, an example of this iterative process between TER calculation and recommendation on mitigation measures is given by the EFSA Scientific Report on the risk assessment of nicosulfuron, one of the two active ingredients used in Winner Top®, which is the PPP addressed in the present study (EFSA 2007). Based on assessed TER and corresponding comparison with established benchmarks, a no-spray buffer zone of 5 meters was used at step 4 because a high risk for macrophytes had been detected in the preceding steps. Positive outcomes were retrieved from this option, thus it became a recommendation for nicosulfuron application. However, as evidenced by parallel modelling work, the no-spray buffer zone should not solve the problem under geoclimatic conditions where run-off is the dominant route of entry into surface water (EFSA, 2007).

1.2.4. PPP effects on non-target organisms

The environmental compartments most affected by PPPs (water and soil) support living communities interacting within complex food webs that contribute to sustain healthy ecosystem functions. Therefore, it is important to evaluate the potentially adverse effects of PPPs on the non-target biota of both compartments as a basis to characterize the environmental hazardous potential of these substances (Abrantes et al., 2009; Pereira et

al., 2009). This is hence a very broad subject that could hardly be properly covered by a single review study in such a venue as the present one. In this way, here the focus will be put on the contamination of the aquatic compartment, more specifically on surface water contamination, which represents a problem of increasing concern worldwide as noted previously. Thus, aspects relating to PPP effects in non-target aquatic organisms will be reviewed. Consistently and considering the PPP used in this study, herbicides will be focused primarily over other PPPs.

Herbicides are used to kill unwanted plants, also called agricultural weeds, but they can affect non-target aquatic organisms as a side-effect (Cedergreen and Streibig, 2005a; Lewis, 1995; Mohr et al., 2007). Non-target aquatic organisms that can be affected by herbicides belong to different trophic and functional levels, ranging from primary producers such as microalgae and macrophytes (Cedergreen and Streibig, 2005a; Lewis, 1995; Mohr et al., 2007) to consumers such as aquatic invertebrates and vertebrates (Novelli et al., 2012; Pisa et al., 2015; Mehler et al., 2008). Since herbicides are designed to act on plants (e.g. photosynthesis interrupting pathways), one should expect much greater effects on non-target flora than on fauna. It is also expected that the most sensitive non-target aquatic organisms are macrophytes and microalgae (major aquatic primary producers) (Wang 1990; Wang 1991). Considering the physiology of these organisms, macrophytes should be more sensitive to systemic herbicides (intake by absorption through leaves and roots) than algae since they are rooted (Wang, 1990). However, there are other factors that may interfere to promote differential sensitivity of organisms to herbicide exposure, such as habitat specificities and behaviour (Carlile, 2006). For example, duckweeds only inhabit the water surface and microalgae are distributed throughout the water column, so the exposure to a given toxic is often different (Wang, 1991, 1990).

Several studies have been performed examining herbicide effects on non-target aquatic organisms (Cedergreen and Streibig, 2005a; Moore et al., 1998; Villarroel et al., 2003), and some examples can be pointed out to illustrate the differential sensitivity of microalgae and macrophytes in particular. Bražėnaitė (2006) exposed *Raphidocelis subcapitata* to pendimethalin (a systemic dinitroaniline herbicide) for 72 h and determined a growth EC_{50} value of 0.052 mg/L. This denoted a higher sensitivity of this species to this particular systemic herbicide compared to macrophytes, which had been tested earlier; Cedergreen & Streibig (2005a) determined a pendimethalin 7 days-EC_{50} of 0.634 ± 0.065 mg/L for *Lemna minor* growth. Turgut & Fomin (2002) evaluated the effects of seventeen pesticides, including pendimethalin, in a representative submersed rooted macrophyte (*Myriophyllum aquaticum*) and they determined a 14 days-EC_{50} values ranging within 10.74-24.13 mg/L for this herbicide. Such a lower sensitivity to pendimethalin compared to *L. minor* evidences that major differences in sensitivity to herbicides may occur even between macrophytes alone. Ferraz et al. (2004) studied the effects of a post-emergence herbicide, propanil in the growth of four algal species and found distinct 72 h-EC_{50} values by more than one order of magnitude: 3.21 mg/L for *Chlorella saccharophila*, 5.98 mg/L for *Chlorella vulgaris*, 0.29 mg/L for *Scenedesmus acutus* and 0.33 mg/L for *Scenedesmus subspicatus*. Pereira et al. (2009) obtained a propanil 96 h-EC_{50} value of 0.023–0.037 mg/L for *R. subcapitata*, evidencing that this species is amongst the most sensitive to the herbicide. These studies suggest that there is also appreciable variation concerning the response of different algae genus and species to a same herbicide. Such a trend was

confirmed by Vidal et al. (2011), who exposed different microalgae species to phenmedipham (a selective systemic phenyl-carbamate herbicide) and found 96 h-EC_{50} values for growth which were distinct by more than one order of magnitude: 0.066 mg/L for *Raphidocelis subcapitata*, 0.481 mg/L for *Chlorella vulgaris* and 0.256 mg/L for *Chlamydomonas pseudocostata.* Despite its systemic action, phenmedipham was as toxic to *R. subcapitata* as it was to the macrophyte *Lemna minor* (Vidal et al. 2011).

In spite of the herbicides' apparent specificity towards producers sharing most metabolic pathways with the target weeds, there are other groups of non-target organisms that also present high sensitivity to these PPPs. Moore et al. (1998) found a propanil 48 h-LC_{50} value of 1.65 mg/L for the microcrustacean *Ceriodaphnia dubia*, which was proved to be the most sensitive among the tested organisms (*Hyallela azteca, Xenopus laevis, Chironomus tentans* and *Pimephales promelas*). This study shows that *C. dubia* can be more sensitive to propanil than some species of algae (see above the data by Ferraz et al. (2004)). *H. azteca*, an epibenthic invertebrate, was nearly four times less sensitive than *C. dubia* (48 h-LC_{50} = 6.58 mg/L). Conversely the benthic invertebrate *C. tentans* was the least sensitive organism tested (48 h-LC_{50} = 17.09 mg/L), overcoming the vertebrates *X. laevis* and *P. promelas* (48 h-LC_{50} = 8.17 and 8.64 mg/L, respectively) and being the single organism tested with clearly higher tolerance by one order of magnitude compared to the most tolerant microalgae tested by Ferraz et al. (2004). Further propanil EC_{50} values for daphnids were obtained in other studies: Villarroel et al. (2003) obtained an immobilization 48 h-EC_{50} value of 5.01 mg/L while Pereira et al. (2009) obtained values within the range 1.8–2.5 mg/L, lower than the equivalent benchmark for some microalgae species (Ferraz et al. 2004). Pereira et al. (2000) applied microbiotest assays and verified that propanil was more toxic to *R. subcapitata* (growth inhibition test) than to *Daphnia magna* (immobilization), to the marine crustacean *Artemia salina* and the freshwater crustacean *Thamnocephalus platyurus* (evaluated parameter: mortality).

The studies presented above suggest that, although some exceptions can be found, the sensitivity of algae and macrophytes to herbicides is generally higher than for other organisms such as benthic invertebrates and fish. In this way, these producer groups should be considered of primary ecological concern when assessing the environmental hazardous potential of herbicides.

1.2.5. Pesticides as a mix of substances

PPPs are formulations/preparations composed by one or more active ingredients/active substances/active principles and a set of other chemicals, also known as inert ingredients. The designations 'formulants' and 'adjuvants' are used to describe the other so-called inert ingredients (Cox and Surgan, 2006; Mesnage et al., 2014; Surgan et al., 2010). The terminology used varies slightly across OECD countries but (OECD, 2008) in general, the a.i. is the one component that acts to control the pests and the adjuvants or formulants are the components supposedly added for purposes other than a pesticidal effect (Surgan et al., 2010).

The use of these so-called inert ingredients on PPPs dates back to eighteenth and nineteenth centuries when pitch, resins, flour, molasses and sugar were used with lime, sulphur, copper and arsenates to improve biological performance (Castro et al., 2014).

Nowadays, the goal of using formulant ingredients is supposedly the same: to use substances that are inactive, when they are used apart, to improve the stability, delivery and effectiveness of the PPP (Castro et al., 2014; Surgan et al., 2010).

The so-called inert ingredients are usually classified according to their use rather than their chemistry. They include surfactants, solvents, etc. (Table 2) (Castro et al., 2014; Marquardt et al., 1998; Simões, 2005; Surgan et al., 2010). For example, surfactants play an important role in the preparation and maintenance of herbicide long-term physical stability, besides they enhance the agrochemical's biological performance (Castro et al., 2014).

Table 2 – Some examples of ingredients other than the a.i.(s) used in PPP formulations (Simões, 2005).

Name	Description		
Solvents and thinners	Dissolve active substances into other substance (mostly used when the active substances are not soluble in water).		
Surfactants	Reduce the surface tension to achieve different functions:	**Wetting agents**	promote the adhesion to the surface of plant organs.
		Dispersing agents	prevent agglomeration of the suspension particles.
		Emulsifying agents	prevent the separation of aqueous and oily phases in the case of emulsions.
		Anti-foam agents and others	used as anti-dust, adhesives, etc.
Inert fillers	Reduce the concentration of a.i. and give consistency, volume and physical form to the formulated product.		

A PPP can be presented in diverse forms such as granular, emulsifiable or wettable (Table 3), depending on the formulants used in the formulation (Cox and Surgan, 2006; CropLife International, 2008), which in turn relates to the solubility and stability characteristics of the a.i.(s). Water soluble a.i.s are generally prepared as aqueous solutions or soluble powder formulations. In contrast, the preparation of formulations with a.i.s of poorer water solubility involves the use of water-miscible organic solvents and other ingredients to solubilize, suspend or disperse the a.i. in an aqueous solution or stable suspension for safe use in agriculture practices (Katagi, 2008; Knowles, 2008).

Table 3 - Some examples of the most common formulation types currently marketed for further mixing with water and application in the field (APVMA, 2014; CropLife International, 2008).

Code	Name	Description
EC	**Emulsifiable concentrate**	"A liquid, homogeneous formulation to be applied as an emulsion after dilution in water."
WP	**Wettable powder**	"A powder formulation to be applied as a suspension after dispersion in water."
SL	**Soluble (liquid) concentrate**	"A clear to opalescent liquid to be applied as a solution of the active constituent after dilution in water. The liquid may contain water-insoluble formulants."
SP	**Soluble powder**	"A powder formulation to be applied as a true solution of the active constituent after dissolution in water, but which may contain insoluble inert ingredients."
SC	**Suspension concentrate**	"A stable suspension of active constituent(s) with water as the fluid, intended for dilution with water before use."
CS	**Capsule suspensions**	"A stable suspension of capsules in a fluid, normally intended for dilution with water before use."
WG	**Water dispersible granules**	"A formulation consisting of granules to be applied after disintegration and dispersion in water."

1.2.6. Regulation of PPPs use in the EU and in Portugal

The recognition of PPPs hazardous potential to human health and the environment by the European Union and European parliament led to deployment of regulations that have become indispensable (EC, 2011, 2009, 1991). The first regulation attempt in this context emerged in 1991 with the Directive 91/414/CEE (EC, 1991) and its main purpose was to impose a re-evaluation of all a.i.s placed on the European market, taking into consideration toxicological and ecotoxicological parameters.

Currently, Directive 91/414/CEE no longer applies and the Regulation (EC) No. 1107/2009 (EC, 2011, 2009; Vieira, 2012) is the major document into force in the EU regarding PPPs. This regulation aims the protection of human and animal health as well as the environment from potential side-effects of PPP, establishing a common action ground for the purposes to all Member-States. The agrochemicals industry has to guarantee that all substances and products placed on the market are not harmful; in other words the a.i.s and formulants or adjuvants composing the formulation must have benefits to plant production and any harmful effects on human and animal health, as well as no unacceptable impacts on the environment. The formulants or adjuvants can only be used

in formulations if they do not belong to Annex lists discriminating hazardous substances (EC, 2009).

PPPs must comply with a set of rules authenticated by the competent authorities before they can be authorized to enter the market. For example, the a.i.s and formulants or adjuvants must be submitted to several toxicological and ecotoxicological studies. Regarding aquatic toxicity studies, mandatory endpoints include LC_{50}/EC_{50} estimation for several non-target representatives of aquatic species, log Pow, bioaccumulation and bioconcentration factors, etc. Environmental fate information such as persistence and degradation rates in different environmental matrices is also required (EC, 2011, 2009; Marrs and Ballantyne, 2004). Each approved PPP must mandatorily have a certified label by the competent authority that works as its "identification card" (EC, 2009; Simões, 2005). Important information about the studies performed during PPP evaluation/approval must displayed on this label. This general information relates to the biological and physico-chemical properties, toxicity and metabolism, environmental behaviour, ecotoxicity and risks for consumers (EC, 2009; Simões, 2005).

In Portugal, the placement of PPPs on the market ultimately depends on Regulation (EC) No. 1107/2009, such as in all other EU countries (DGAV, 2015a; Vieira, 2012). The initial assessment of the PPP based on international criteria is performed in DGAPF (Divisão de Gestão e Autorização de Produtos Fitofarmacêuticos), and the competent authority for their approval in national territory is DGAV - Direção Geral de Alimentação e Veterinária (DGAV, 2015a). The PPPs list with authorized sale in Portugal can be accessed on the DGAV website (DGAV, 2015b).

1.2.7. Formulation omissions

The so-called inert ingredients added to the formulations are frequently omitted (or they are not chemically identified) on the product label (Cox and Surgan, 2006; Surgan et al., 2010) by the manufacturing companies. Most times they are designated as inert ingredients and their total percentage is mentioned on the label but there is no reference to their individual chemical identity. In current language, the term "inert" refers to something that is physically, chemically or biologically inactive. This designation can cause a misunderstanding to consumers, assuming it may be water or other harmless ingredients (EPA, 2015). Theoretically, the a.i. is the responsible for preventing, destroying, repelling, or mitigating any pest, as already mentioned above (Cox et al., 2006), and it is usually subjected to greater scrutiny tests when compared to the other ingredients or the whole marketed products (Cox and Surgan, 2006; EC, 2009; Surgan et al., 2010). The available information about the toxicity of the entire product (PPP formulation) is limited, i.e. the interaction between a.i.s and the so-called inert ingredients is not considered and this may compromise the evaluation of the environmental toxicity of the PPP. The regulation only requires the disclosure of the a.i.s and formulants or adjuvants proven to represent an environmental hazard *per se* (please see above) (EC, 2009). Nevertheless, there are published studies with representative biota e.g. bacteria, microalgae, plants, cladocerans, fish, that indicate the so-called inert ingredients may enhance the toxicity of PPPs, suggesting that toxicity tests based only in the a.i.s and formulants/adjuvants *per se* are

inadequate (Beggel et al., 2010; Cedergreen and Streibig, 2005b; Cox and Surgan, 2006; Demetrio et al., 2012; Joly et al., 2013; Marquardt et al., 1998; Schmuck et al., 1994; Vidal et al., 2011). The so-called inert ingredients can hence be hazardous for environmental health and for humans (Cox and Surgan, 2006; Mesnage et al., 2014). Moreover, these "inert ingredients" frequently correspond to the major fraction in the PPP formulations (Marquardt et al., 1998; Selectis, 2012; Surgan et al., 2010). For example, in Winner Top®, formulants comprise 72.9% of the whole formulation. In addition, the so-called inert ingredients can compromise the integrity and utility of pesticide risk indicators (PRI) because they cannot be considered in any analyses if there is no information in the product label about their chemical identity (Surgan et al., 2010). PRI provide information about the risk of damage to terrestrial and aquatic environments, as well as human health due to pesticide exposure and derived toxicity (OECD, 2008; Surgan et al., 2010).

1.2.8. Discovery and development of new PPPs

Nowadays it's remarkable that the pressure caused by government authorities and consumer organizations have been forcing the development of a wide range of product formulation types, additives and even new technologies to enable PPP formulations (Castro et al., 2014; Knowles, 2008). This is required to maximize PPPs efficacy, to prevent unfavourable environmental contamination by them and by their degradation products (Castro et al., 2014; Katagi, 2008) and to reduce potential impacts on human health and on non-target organisms (Castro et al., 2014; Damalas and Eleftherohorinos, 2011). In other words, there is an increasing need of producing safer, more convenient to use, more effective and less hazardous PPPs following general "green chemistry principles" (Anastas and Warner, 1998). These "environmentally-friendly" alternative agrochemical products, expected to have faster environmental degradation (Damalas and Eleftherohorinos, 2011; Fan et al., 2014; Knowles, 2008), should be based on: the exploitation of the biocide properties of natural products (Cantrell et al., 2012; Dayan et al., 2009; Singh et al., 2013); the improvement of drift technologies or drift control agents added to the formulation (Felsot et al., 2011); the development of better product delivery systems to avoid losses by evaporation, leaching, degradation and volatization of the a.i.s (Singh et al., 2013); the design of new surfactants (Castro et al., 2014; Knowles, 2008; Straub et al., 2014) or greener solvents (Fan et al., 2014) with lower environmental impacts. These are up-to-date challenges for researchers and agrochemical companies since they may provide suitable answers to new registration requirements (Cantrell et al., 2012; Castro et al., 2014; Dayan et al., 2009; EC, 2009). However, at least while the novelties are not fully incorporated in the market, environmental contamination by PPPs is still a reality requiring the best attention, this including the role of formulants in the overall environmental toxicity of PPPs.

1.3. The experimental models used in the present study

1.3.1. Winner Top®

The selection of an herbicide focused in the present work was driven primarily on statistics about PPP sales. Although fungicides are the PPPs with the best sale records, a large part of these sales is due to sulphur (please see sections 1.2.1.2 and 1.2.1.3 above), which necessarily narrows the range of options for further selection – the representativeness of any fungicide other than sulphur would be low. Herbicides as the representatives of the second most consumed PPPs were then selected for this work. Then, Winner Top® was further selected following a thorough analysis of the known composition of all herbicide formulations available in the Portuguese market (so those we can gain access easily). The selection of this commercial formulation was based mainly on its convenient composition. It combines two a.i.s (nicosulfuron and terbuthylazine) plus undisclosed formulants. By reducing the foreseen mixtures toxicity analysis (see section 1.4 for the workplan) to a binary system, a necessary simplification in the applied predictive approaches was achieved for a better focus on the most innovative components of the study. Another reason for the selection was the lack of information in literature regarding the environmental toxicity of this formulation and the limited information available regarding the active ingredients, which would configure the study as an important contribute to the ecotoxicological database on the chemicals involved.

Winner Top® is an herbicide marketed in Portugal by Selectis® which has been used on maize cultures to combat the annual grass weeds and dicotyledonous. It is a 2-way formulation using 250 g/L or 25.4% (w/w) terbuthylazine and 16.75 g/L or 1.7% (w/w) nicosulfuron as active ingredients. The formulation is an oil dispersion and, in addition to the 2 a.i.s, the so-called inert ingredients, which are not disclosed in the label, make up 72.9% (w/w) of the formulation. Nicosulfuron is a sulfonylurea and terbuthylazine is a 1,3,5-triazine (DGAV, 2015a; Selectis, 2016, 2012). Some of the most relevant physical and chemical properties of nicosulfuron and terbuthylazine are presented in Table 4.

This post-emergence herbicide has a systemic and residual (biocidal effects persisting through time) action, being absorbed by roots and leaves of the plants. It should be used on maize cultures with 4-6 leaves, when pest weeds have up to 3-5 leaves. The recommended application dose is 2.5 L/ha for most susceptible species or in soils with high organic matter content and 3 L/ha for moderately susceptible species (Table 5). A pickle should be prepared for pesticide application (200-400 L/ha) once a year. The herbicide effects can be recognized 7-10 days after the application through the blocking of weeds growth. It is considered a very effective herbicide (Selectis, 2012).

Table 4 - Identity and physico-chemical properties of nicosulfuron and terbuthylazine. The information was synthesized from the PPDB database (Lewis et al., 2016), which is a wide-range database characterizing PPPs banned and used in Europe considering the EU regulation applicable. The figures with the chemical structures were retrieved from the PubChem Substance and Compound database through the unique chemical structure identifier CID 73281 (nicosulfuron) and 22206 (terbuthylazine).

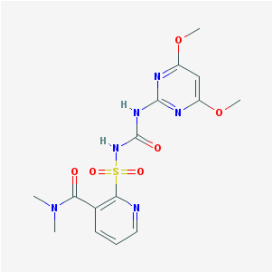
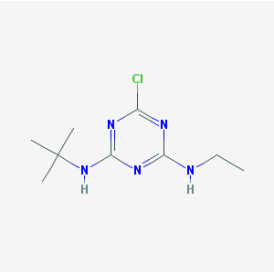
	Nicosulfuron	**Terbuthylazine**
Molecular structure	(a) 	(b) 
IUPAC name	2-[(4,6-dimethoxypyrimidin-2-ylcarbamoyl)sulfamoyl]-*N,N*-dimethylnicotinamide	*N*2-tert-butyl-6-chloro-*N*4-ethyl-1,3,5-triazine-2,4-diamine
CAS name	2-[[[[(4,6-dimethoxy-2-pyrimidinyl)amino]carbonyl]amino]sulfonyl]-*N,N*-dimethyl-3-pyridinecarboxamide	6-chloro-*N*-(1,1-dimethylethyl)-*N*'-ethyl-1,3,5-triazine-2,4-diamine
CAS number	111991-09-4	5915-41-3
Molecular Formula	$C_{15}H_{18}N_6O_6S$	$C_9H_{16}ClN_5$
Molecular weight	410.41 g/mol	229.71 g/mol
Melting point	145°C	176°C
Solubility (in water) at 20°C	7500 mg/L (high)	6.6 mg/L (low)
Solubility (in organic solvents) at 20°C	Acetone: 8900 mg/L Dichloromethane: 21300 mg/L Methanol: 400 mg/L Ethyl acetate: 2400 mg/L	Acetone: 41000 mg/L Dichloromethane: 9800 mg/L Methanol: 12000 mg/L Ethyl acetate: 410 mg/L
Octanol-water partition coefficient at pH 7, 20°C	0.61 (low)	3.4 (high)
Vapour pressure at 25°C (mPa)	8.00 X 10^{-7} (non-volatile)	0.12 (non-volatile)
Henry's law constant at 25°C (Pa m^3 mol^{-1})	1.48 X 10^{-11} (non-volatile)	3.24 X 10^{-3} (non-volatile)
GUS leaching potential index	3.25 (high leachability)	3.07 (high leachability)
Physical state	White powder or colourless crystals	White crystalline powder

Table 5 – Recommended application doses for Winner Top®, with representation of the inherent doses of active ingredients.

Formulation		**Recommended application doses**	
Winner Top®		**2.5 L** Winner Top/ha	**3 L** Winner Top/ha
Nic.	16.75 g/L	41.88 g/ha	50.25 g/ha
Terb.	250 g/L	625 g/ha	750 g/ha

The modes of action of both active ingredients are well known and characterized. Nicosulfuron prevents the growth of the plant by blocking the plant amino acid synthesis through the inhibition of acetohydroxyacid synthase (AHAS) enzymes. Terbuthylazine inhibits the photosynthesis by acting as a photosystem II blocker (DGAV, 2015a; Lewis et al., 2016). Concerning environmental fate, terbuthylazine is moderately persistent in soil with a DT_{50} = 75.1 (lab at 20°C) and 22.4 (field) days and nicosulfuron is not considered persistent in soil since it has a DT_{50} = 16.4 (lab at 20°C) and 19.3 (field) days under aerobic degradation (Lewis et al., 2016; Selectis, 2012). Nicosulfuron degrades moderately fast in the water-sediment interphase (DT_{50} = 41.5 days) and it is stable in the water phase (DT_{50} = 65 days); terbuthylazine degrades moderately fast in the water-sediment interphase (DT_{50} = 70 days) and in the water phase (DT_{50} = 6 days). In addition, nicosulfuron degrades faster via foliar route (DT_{50} = 5 days) and under acid conditions (DT_{50} = 15 days at pH 5) mainly by chemical hydrolysis (Lewis et al., 2016). There is no information available regarding the environmental fate of Winner Top® as a whole product.

1.3.2. The target weeds and treatment strategies

The most susceptible species to Winner Top® are *Portulaca oleracea, Amaranthus* spp., *Poa annua*, *Lolium rigidum, Solanum nigrum, Polygonum persicaria, Datura stramonium, Abutilon theoprasti, Chamaemelum mixtum, Echinochloa crus-galli, Anagallis arvenses, Chenopodium* spp., *Rumex* spp., *Raphanus raphanistrum, Setaria viridis, Setaria verticillata, Setaria pumilla, Sonchus oleraceus* and *Sorghum halepense.* Moderately susceptible species are *Polygonum lapathifolium* and *Sorghum halepense.*

The target weed selected for the 2nd part of this study (see section 1.4) is one of the main targets of Winner Top® as well recognised in the product's label - *Portulaca oleracea* (Figure 4). The choice of this species was facilitated by its suitable features, in particular the fact that it is very common and easy to find as seeds since it is consumed in Mediterranean salads; it is of a relatively small size, which allows easy handling in the laboratory; and it can be cultivated all over the year.

Portulaca oleracea (common name purslane; "beldroega" in Portuguese) is an annual herbaceous plant of the family Portulacaceae (Figure 4). This weed is a principal weed affecting a wide range of crops (Chauhan & Johnson, 2009; Cabi, 2015; Kafi & Rahimi, 2011), including maize, rice, wheat, cotton, sugarcane, tea and vegetables (Chauhan and Johnson, 2009; Haar and Fennimore, 2003). *P. oleracea* presents succulent stems that may grow erect, semi-erect or prostate (Grieve & Suarez, 1997; Chauhan & Johnson, 2009) depending on the available light. Its life cycle can be completed in 2-4 months in both tropical and temperate regions and numerous seeds (as many as 10 000) can be produced by a single plant. The stem fragments can also produce roots and this is another form of plant propagation (Chauhan and Johnson, 2009).

Figure 4 – *Portulaca oleracea*.

Although this species is considered a weed, it is used as a medicinal plant. Its aerial parts have pharmacological properties on swelling and pain relief, and they are also used as an antiseptic (Chan et al., 2000; Zhou et al., 2015). Some recent studies also show that *P. oleracea* is rich in omega-3 fatty acids, which are important in preventing heart attacks and strengthening the immune system (Kafi and Rahimi, 2011; Zhou et al., 2015). Furthermore, purslane has been widely used as current vegetable for human consumption mostly in salads and soups in various Mediterranean and Central American countries (Grieve and Suarez, 1997; Zhou et al., 2015).

1.3.3. Non-target aquatic organisms in general and *Lemna minor* in particular

Winner Top® can reach waterbodies by processes such as run-off and spray drift, and it is a very toxic compound for aquatic species, causing long-term effects in the aquatic environment; this lead to its EC risk classification as dangerous for the environment (Ashauer et al., 2011; Selectis, 2012). Only the a.i.s of Winner Top® were already analysed for their aquatic ecotoxicity. As to our knowledge, the aquatic toxicity of the formulation is not yet covered in the literature. Nicosulfuron's concentrations inducing 50% reduction on an indicative physiological variable (EC_{50}) measured following testing with different aquatic species during a certain exposure period are presented in Table 6. A similar collection is presented in Table 7 for terbuthylazine.

Table 6 - Nicosulfuron ECs_{50}/LCs_{50} obtained in literature for different freshwater aquatic species with reference to the focused variable and exposure period (duration). When available, 95% confidence intervals were represented within brackets.

Species	Endpoint	Test duration	EC_{50} /*LC_{50}	References
Microalgae				
Raphidocelis subcapitata	Growth	72 h	>1000 µg/L	(Mohammad et al., 2005)
	Growth	96 h	1.4315 mg/L	(Ma et al., 2006)
Oscillatoria limnetica	Growth	11 days	2.4 mg/L	(Leboulanger et al., 2001)
Scenedesmus obliquus	Growth	96 h	4.6294 mg/L	(Ma, 2002)
Scenedesmus quadricauda	Growth	96 h	3.7 mg/L	(Ma et al., 2004)
Chlorella pyrenoidosa	Growth	96 h	2.200 mg/L	(Ma, 2002)
Chlorella vulgaris	Growth	96 h	4.3311 mg/L	(Ma et al., 2002)
Anabaena flos-aquae	Growth	72 h	7.8 mg/L	(Lewis et al., 2016; Selectis, 2012)
Crustaceans				
Daphnia magna	Immobilisation	48 h	90.0 mg/L	(Lewis et al., 2016; Selectis, 2012)
Aquatic plants				
Lemna sp.	Growth	7 days	14.5 µg/L	(Mohammad et al., 2005)
Lemna gibba	Biomass	7 days	0.002 mg/L	(Lewis et al., 2016; Selectis, 2012)
Fish				
Lepomis macrochirus	Mortality	96 h	> 1000000 µg/L *	(U.S. EPA, 1992)
Oncorhynchus mykiss	Mortality	96 h	65.7 mg/L *	(Lewis et al., 2016; Selectis, 2012)
Amphibian				
Xenopus Laevis	Malformation	4 days	3.1 (2.6-3.6) mg/L	(Fort et al., 1999)

Table 7 - Terbuthylazine ECs_{50}/LCs_{50} obtained in literature for different freshwater aquatic species with reference to the exposure time. When available, the 95% confidence intervals or standard deviation associated to each benchmark were shown within brackets.

Species	Endpoint	Test duration	EC_{50}/ LC_{50}*	References
Algae				
Raphidocelis subcapitata	Growth	24 h	33 (± 0.07) µg/L	(Pérez et al., 2011)
	Growth	48 h	20 (± 0.07) µg/L	(Pérez et al., 2011)
	Growth	72 h	24 (± 0.08) µg/L	(Pérez et al., 2011)
	Growth	48 h	595 (± 272) µg/L	(Munkegaard et al., 2008)
	Growth	72 h	9 (6-15) µg/L	(Sbrilli et al., 2005)
	Growth	72 h	0.012 mg/L	(Lewis et al., 2016)
	Growth	72 h	55 µg/L	(Cedergreen and Streibig, 2005a)

	Growth	72 h	0.028 mg/L	(Selectis, 2012)
Scenedesmus subspicatus	Growth	72 h	0.016 mg/L	(Nitschke et al., 1999)
Crustaceans				
Daphnia magna	Immobilization	48 h	21200 (16000-26800) µg/L	(Lewis et al., 2016; U.S. EPA, 1992)
	Immobilization	96 h	50900 (36000-85600) µg/L	(U.S. EPA, 1992)
Aquatic plants				
Lemna minor	Growth	7 days	157 (± 18) µg/L	(Munkegaard et al., 2008)
	Growth	7 days	0.23 mg/L	(Nitschke et al., 1999)
	Growth	12 days	≈ 150 µg/L	(Cedergreen and Streibig, 2005a)
	Growth	12 days	100<EC_{50}<250 µg/L	(Cedergreen and Streibig, 2005a)
	Growth	14 days	153 µg/L	(Cedergreen et al., 2004)
	Growth	14 days	182 µg/L	(Cedergreen et al., 2004)
	Growth	14 days	111 µg/L	(Cedergreen et al., 2004)
	Growth	14 days	40 µg/L	(Cedergreen et al., 2004)
	Growth	7 days	105 µg/L	(Cedergreen and Streibig, 2005b)
Lemna gibba	Biomass	7 days	0.0128 mg/L	(Lewis et al., 2016)
		14 days	16 (12-20) µg/L	(U.S. EPA, 1992)
	Growth	14 days	0.412 mg/L	(Selectis, 2012)
Fish				
Danio rerio		96 h	13 (± 1.81) mg/L	(Pérez et al., 2013)
Oncorhynchus mykiss	Mortality	96 h	2.2 mg/L *	(Lewis et al., 2016; Selectis, 2012)
	Mortality	96 h	3400 (2400-4700) µg/L *	(Lewis et al., 2016; U.S. EPA, 1992)
Lepomis macrochirus	Mortality	96 h	7500 (5600-10000) µg/L *	(U.S. EPA, 1992)

As discussed earlier, since Winner Top® is an herbicide, greater effects are expected on flora than on fauna species. Tables 6 and 7 confirm the higher sensitivity to the a.i. by macrophytes and algae, especially for terbuthylazine, compared to other organisms. This provided the necessary support for the selection of these non-target organisms to develop the present study, which is committed to the establishment of environmental safety principles ruling PPPs development. Furthermore, these selected non-target organisms represent primary producers of freshwater ecosystems and collect different levels of physiological complexity, which concomitantly allows covering of different toxicant uptake pathways. This selection hence included two species of microalgae, *Raphidocelis subcapitata* and *Chlorella vulgaris*, and two species of macrophytes, the duckweeds *Lemna minor* and *Lemna gibba.* In addition, the exposure to toxics differs between these two

primary producers since duckweeds inhabit the water surface only and microalgae are distributed throughout the water column, thus they have no surface that can be exempted from exposure (Wang, 1991, 1990). While the four species were used in the first stage of the study (chapter 2), only one of them was selected for the second stage of the study (chapter 3) due to the enlarged complexity of the experimental design involved. The selected species at this second stage was *L. minor* due to its high sensitivity to the tested toxics; considering logistic issues, *L. minor* was preferred over *L. gibba* given its smaller size allowing easier handling in the laboratory.

Raphidocelis subcapitata (formerly *Selenastrum capricornutum* and *Pseudokirscheneriella subcapitata*) is a green unicellular freshwater microalgae of the family Selenastraceae. It is a sensitive planktonic species, which is typically used in standard toxicity tests as a representative of primary producers. Its cells are curved, twisted and normally solitary (Figure 5) (OECD, 2006a). *R. subcapitata* reproduces asexually by autospore formation (generally 2-4 per sporangium). The autospores are released by transverse to longitudinal rupture of parental cell wall (Guiry and Guiry, 2016). This algal species is a recommended standard species for algal toxicity tests. It is easy to maintain in the laboratory and cell density measurements can be easily performed in an electronic particle counter or under a microscope using simple counting chambers (OECD, 2006a).

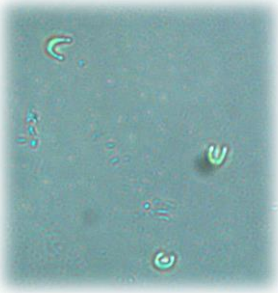


Figure 5 – *R. subcapitata* cells under the microscope (magnification: 200x).

Chlorella vulgaris is a freshwater planktonic microalgae of the family Chlorellaceae (Figure 6) (Safi et al., 2014; Yamamoto et al., 2005, 2004). It reproduces asexually by autosporolation. When the new cells are mature, the mother cell wall ruptures for liberation of the daughter cells comprised by their own wall cells - generally 2-4 daughter cells per autosporangium (Safi et al., 2014; Yamamoto et al., 2004). This species is of easy culturing in the laboratory (Yamamoto et al., 2004) and it's of wide use in toxicity studies just like *R. subcapitata* (Leboulanger et al., 2001; Lewis, 1995; Ma et al., 2002).

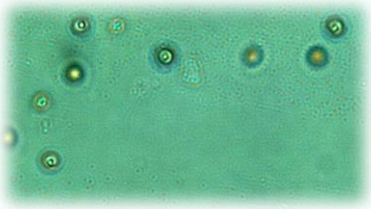


Figure 6 - *C. vulgaris* cells under the microscope (magnification: 200x).

Lemna minor and *Lemna gibba* (Figure 7) are two species of duckweed plants of the family Lemnacea. These species have a simple structure composed of two parts: a floating or partly submerged frond and a very thin root emanating from the centre of the lower surface of each frond (OECD, 2006b; Wang, 1990). Commonly, these vascular plants are colonial and they form aggregates of two or more fronds (Wang, 1990). They reproduce asexually by vegetatively producing new fronds and their generation time is short (OECD, 2006b; Wang, 1990). Duckweed plants are widely distributed in the world from freshwater to brackish systems, meaning that they are relevant to many aquatic environments such as lakes, streams and effluent waterways (Wang, 1990). They constitute food for small aquatic animals including fish. These plants also provide shelter and shade for fish and they can serve as a physical support for several small invertebrates (Wang, 1991).

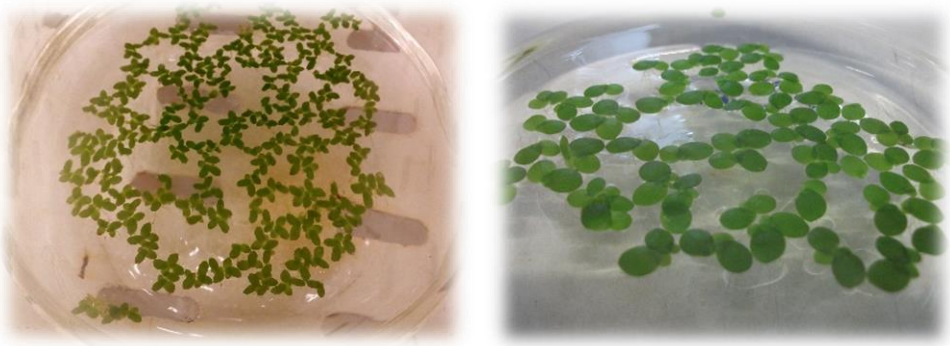


Figure 7 - *Lemna minor* (left-hand panel) and *Lemna gibba* (right-hand panel) cultured in the laboratory.

Lemna spp. has been recommended as standard test organism. It has a set of appropriate characteristics such as small size (*L. minor*: 2-4 mm across; *L. gibba*: 3-5 mm across), simple structure and short generation time. It is of very easy maintenance and handling in the laboratory and for conducting toxicity tests (OECD, 2006b; Wang, 1990). *Lemna* spp. are especially suitable for testing herbicide pollution in the aquatic environment (Wang, 1990). There are studies indicating duckweeds as tolerant to environmental toxicity and they were even designated as the "carp" of plant species because of their tolerance to pollution (Kanoun-Boulé et al., 2009; Khellaf and Zerdaoui, 2010; Prasad et al., 2001; Wang, 1990). Therefore, some studies (Ansal et al., 2010; Ansari and Khan, 2008; Del-Campo Marín and Oron, 2007; Khellaf and Zerdaoui, 2010) indicate these plants as suitable for bioremediation through the removal of low concentrations of pollutants from water. Conversely, there are other studies suggesting that duckweeds are very sensitive to pollutants, hence arguing on their suitability as bioindicator species of water pollution (Garg and Chandra, 1994; Hegazy et al., 2009; Nasu and Kugimoto, 1081).

1.4. Objectives and structure of the dissertation

Following the general rationale described in section *1.1. Introductory note*, several specific aims were set for the present studies and they were as follows.

i) To contribute with relevant ecotoxicological information on short-term effects of the herbicide Winner Top® and its a.i.s (nicosulfuron and terbuthylazine) to different non-target freshwater species, the macrophytes *Lemna minor* and *Lemna gibba,* and the microalgae *Raphidocelis subcapitata* and *Chlorella vulgaris*.

ii) To compare the toxicity of each of the two a.i.s with that of the commercial formulation Winner Top® and that of a customized mixture of the a.i.s respecting their ratio in the commercial formulation.

iii) To assess whether unknown formulants enhance the overall toxicity of Winner Top® by comparing its effects with the effects induced by a

customised mixture of terbuthylazine and nicosulfuron respecting the formulation ratio used in Winner Top®.

iv) To study the response surface by a non-target organism (*Lemna minor*) following exposure to the combination between the a.i.s for spotting deviations from the reference models of mixture toxicity, with particular interest on antagonism; antagonistic mixtures of the a.i.s can eventually be used to rule the formulation of eco-friendlier Winner Top® equivalents.

v) To test the efficacy of eco-friendlier mixtures of a.i.s as spotted to meet objective iv, using a Winner Top®' target species (*Portullaca oleracea*).

The first mentioned objective was set since neither there are ecotoxicological data on the formulation nor on the likelihood of an interactive behavior between Winner Top®'s a.i.s was found in the literature. It therefore allowed us to complete the available body of knowledge about the ecotoxicity of nicosulfuron, terbuthylazine and Winner Top® towards some aquatic representative species. The evaluation of the toxicity of each of the a.i., that of the commercial formulation Winner Top® and that of a customized mixture of the a.i. respecting their ratio in the commercial formulation (objective ii) allowed us to compare the toxicity of the a.i.s and to rank the sensitivity of the non-target organisms selected, which was critical information to better handle the second part of the study. Objective iii was logically set because the so-called inert ingredients constitute a significant part (72.9%) of the commercial product, this supporting concerns on the possible modification of the toxicity of the herbicidal ingredients driven by these formulants. And in fact, this likely effect of formulants is an up-to-date topic in specialized discussion arenas on pesticide regulation and ecotoxicology. Finally, objectives iv and v allowed us to understand if the manipulation of ratios between the a.i.s in the formulation could be an innovative solution to reduce the environmental toxicity of the PPP. The ultimate goal was to find an alternative formulation of the a.i.s that shows an eco-friendlier behaviour considering standard non-target indicators while keeping its efficacy against target weeds.

In order to address all of these challenges embedded in the planned studies, the dissertation was divided in **four chapters**:

The present chapter (**chapter 1**) is essentially a literature review covering all topics involved in this work. It started with an introductory note immediately establishing the general context of the study as well as noticing the way the work was organized. Following on this introductory note, the body of knowledge available on PPP definition, classification, development, use, regulation and environmental effects was revised. The experimental models used in this study, namely the PPP Winner Top®, the target weed species *Portulaca oleracea* and the non-target organisms *Raphidocelis subcapitata, Chlorella vulgaris, Lemna minor* and *Lemna gibba* were additionally characterized.

The next two chapters organize the experimental work sequentially and were built following the specific layout commonly used in journal articles. In fact, they constitute two manuscripts, in preparation for submission or already submitted to specialized

international peer-review journals. **Chapter 2** regards the ecotoxicological assessment of the herbicide Winner Top® and its a.i.s using standard non-target organisms (*L. minor, L. gibba, R. subcapitata* and *C.vulgaris)* and discussed the role of the unknown formulants in the overall toxicity of the PPP product. **Chapter 3** details the effects of the a.i.s, singly and as mixtures, in the non-target species *Lemna minor* and reports the efficacy against a target weed of eco-friendlier mixtures.

Finally, final remarks on the findings and an integrative discussion of all results are presented in **chapter 4**.

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CHAPTER 2 - Ecotoxicological assessment of the herbicide Winner Top® and its active ingredients – are formulants truly inert?

2.1. INTRODUCTION

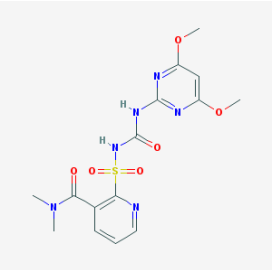
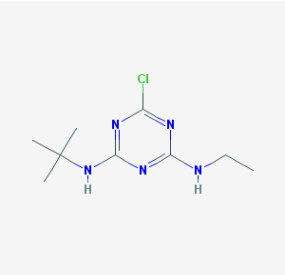
Plant Protection Products (PPPs) are extensively used in agriculture to keep agricultural production rates (Carlile, 2006; Fuentes et al., 2013). Following their application they can reach surface water through different transport pathways and PPP residues have been found frequently in this environmental compartment (e.g. Abrantes et al., 2010, 2009; DeLorenzo et al., 2001). Therefore, PPPs represent a putative environmental hazard and their toxic potential over non-target organisms has been addressed frequently in the literature (e.g. Cedergreen and Streibig, 2005; Pereira et al., 2009; Vidal et al., 2011).

PPPs are formulations composed by one or more active ingredients (a.i.s) and a set of other chemicals, also known as inert ingredients. The designations 'formulants' and 'adjuvants' are used to describe the so-called inert ingredients (Cox and Surgan, 2006; Mesnage et al., 2014; Surgan et al., 2010), typically added to improve PPP efficacy, stability and delivery of the active ingredients to their target (Castro et al., 2014; Surgan et al., 2010).

An up-to-date controversy is the role of the so-called inert ingredients in the environmental toxicity of the PPP. Regulation demands the disclosure of a.i.s and formulants or adjuvants proven to represent an environmental hazard *per se*, but disregards their effects within the formulation, i.e. the interaction between formulation components (EC, 2009). In this way, a given formulant or adjuvant can be environmentally non-toxic when tested alone, hence immediately becoming exempted from disclosure requirements. However, this formulant/adjuvant was added for a reason to the PPP recipe, typically because it can interact with other components constituting the PPP mixture to improve its overall efficacy against the target weed. It is then reasonable to hypothesize that such interactions may also play a role in the toxicity of the PPP to non-target environmental receptors.

In the present study, the commercial herbicide Winner Top® was selected to address the above problematic. Winner Top® is a 2-way formulation using terbuthylazine and nicosulfuron as active ingredients (a.i.s). The other ingredients are not individually identified on the PPP label. The formulation is classified as an oil dispersion and, taking into account the percentage of the a.i.s present in the formulation (terbuthylazine 25.4% w/w and nicosulfuron 1.7% w/w), inert ingredients constitute 72.9% w/w of the product. Winner Top® has been used on maize cultures to combat annual grass weeds and dicotyledonous weeds (e.g. *Portulaca oleracea, Amaranthus* spp., *Poa annua*). Its application should be performed after the emergence of the weeds (post-emergence herbicide) and its action is systemic by being distributed throughout the plant after being taken up through the roots and leaves, and residual by lasting in the long term. Nicosulfuron and terbuthylazine belong to the sulfonylureas and 1,3,5-triazines chemical groups, respectively (DGAV, 2015a, 2015b; Selectis, 2016, 2012). Some of their physical and chemical properties are presented in Table 1.

Table 1 - Identity and physico-chemical properties of nicosulfuron and terbuthylazine. The information was synthesized from the PPDB database (Lewis et al., 2016), which is a wide-range database characterizing PPPs banned and used in Europe considering the EU regulation applicable. The figures with the chemical structures are freely available in the PubChem Substance and Compound database through the unique chemical structure identifiers CID: 73281 (nicosulfuron) and 22206 (terbuthylazine).

	Nicosulfuron	**Terbuthylazine**
Molecular structure		
IUPAC name	2-[(4,6-dimethoxypyrimidin-2-ylcarbamoyl)sulfamoyl]-*N*,*N*-dimethylnicotinamide	*N*2-tert-butyl-6-chloro-*N*4-ethyl-1,3,5-triazine-2,4-diamine
CAS name	2-[[[[(4,6-dimethoxy-2-pyrimidinyl)amino]carbonyl]amino]sulfonyl]-*N*,*N*-dimethyl-3-pyridinecarboxamide	6-chloro-*N*-(1,1-dimethylethyl)-*N*'-ethyl-1,3,5-triazine-2,4-diamine
CAS number	111991-09-4	5915-41-3
Molecular Formula	$C_{15}H_{18}N_6O_6S$	$C_9H_{16}ClN_5$
Molecular weight	410.41 g/mol	229.71 g/mol
Melting point	145°C	176°C
Solubility (in water) at 20°C	7500 mg/L (high)	6.6 mg/L (low)
Octanol-water partition coefficient at pH 7, 20°C	0.61 (low)	3.4 (high)
Vapour pressure at 25°C (mPa)	8.00 X 10^{-7} (non-volatile)	0.12 (non-volatile)
Henry's law constant at 25°C (Pa m^3 mol^{-1})	1.48 X 10^{-11} (non-volatile)	3.24 X 10^{-3} (non-volatile)
GUS leaching potential index	3.25 (high leachability)	3.07 (high leachability)
Physical state	White powder or colourless crystals	White crystalline powder

The modes of action of both a.i.s are well known and characterized. Nicosulfuron prevents the growth of the plant by blocking the plant amino acid synthesis through the inhibition of acetohydroxyacid synthase (AHAS) (DGAV, 2015a; Lewis et al., 2016), an

enzyme whose site of action exhibits affinity towards different classes of herbicides such as imidazolinones and sulfonylureas (Duggleby et al., 2008; McCourt et al., 2006; Singh et al., 1988). This enzyme only exists in microorganisms and plants, and that is why these compounds are deemed selective, very potent and apparently nontoxic to animals (Duggleby and Pang, 2000; Duggleby et al., 2008; McCourt et al., 2006). Terbuthylazine inhibits the photosynthesis by acting as a photosystem II blocker (DGAV, 2015a; Lewis et al., 2016). The use of herbicides whose mechanism of action is as photosynthesis inhibitors comes from long ago (Pfister and Arntzen, 1979; Powles, 1984). Photosystem II blockers belong to chemical groups such as amides and triazines and are generally assumed selective to photosynthetic organisms (Pfister and Arntzen, 1979).

Neither ecotoxicological data for the formulation nor information on the likelihood of an interactive behaviour between the a.i.s was found in the literature. Furthermore, the so-called inert ingredients constitute a significant part (72.9%) of the product; this evidence supports concerns on a possible modification of the toxicity of the herbicidal ingredients driven by these formulants. In this context, the aim of the present study was to thoroughly characterize the aquatic toxicity of Winner Top®. Implicitly, but not of lessen importance was the goal of assessing whether unknown formulants can significantly contribute to the overall toxicity of the product, i.e. whether inert ingredient are as inert as they should be from an environmentally precautionary point of view. The toxicity of each of the two a.i.s, that of the commercial formulation Winner Top®, and that of a customized mixture of the a.i.s respecting their ratio in the commercial formulation were compared for the purposes.

Standard non-target organisms from the aquatic compartment were judiciously selected to address these aims. In this way, two microalgae species *(Raphidocelis subcapitata* and *Chlorella vulgaris)* and two macrophyte species (*Lemna minor* and *Lemna gibba*), both primary producer representatives, were used as the most direct non-target aquatic recipients for a product with herbicidal properties. At this stage we hypothesized that macrophytes would show higher sensitivity than microalgae since the selected formulation is of systemic action. Although both microalgae species and both macrophyte species are interchangeably accepted as standard test species, differences in sensitivity have been frequently noticed. As examples, the study by Vidal at al. (2011) can be highlighted, evidencing a difference by 7.3 fold between *R. subcapitata* and *C. vulgaris* in sensitivity to the herbicide phenmedipham; and *Lemna minor* has been found more tolerant than *Lemna gibba* to some metals (Dvorák et al., 2012) and some organic solvents (Cowgill et al., 1991). These inconsistencies motivated us to test two algae and two macrophytes, so that a more comprehensive insight could be provided on the ecotoxic potential of the tested chemicals/compounds.

2.2. MATERIAL AND METHODS

2.2.1 Chemicals

The chemicals used in the toxicity bioassays were the herbicide Winner Top®, marketed in Portugal by Selectis® as a concentrated suspension with 16.75 g/L nicosulfuron and 250 g/L terbuthylazine (Selectis®, Portugal; Figure 1), and its a.i.s Terbuthylazine ($C_9H_{16}ClN_5$, CAS No.5915-41-3; Pestanal®, Sigma-Aldrich®, Steinheim) and Nicosulfuron ($C_{15}H_{18}N_6O_6S$, CAS No. 111991-09-4; Pestanal®, Sigma-Aldrich®, Steinheim). Stock solutions were prepared immediately before each assay by dissolving the a.i. or diluting the pesticide formulation in distilled water or in each test medium depending on observed solubility constraints. Unless otherwise noticed, concentrations always refer to the active ingredients, even when addressing assays with the commercial formulation.

Figure 7 – Commercial formulation Winner Top® 1L.

2.2.2. Test organisms

2.2.2.1. *Raphidocelis subcapitata* and *Chlorella vulgaris*

Unialgal cultures of *R. subcapitata* and *C. vulgaris* were cyclically maintained in the laboratory in sterilized Woods Hole MBL medium (Stein, 1973), at 20 ± 2°C with a $16h^{Light}$:$8h^{Dark}$ photoperiod (light intensity: ≈2000 LUX) and permanent aeration to prevent cell clumping at the bottom of the culture vessel. Once a week the cultures were renewed under a sterilized environment (flame assisted laminar flow chamber) by spiking freshly prepared medium with a healthy inoculum preserved following harvesting, normally in the week before from the grown culture. These inocula were made in 100-mL Erlenmeyer vessels filled with ca. 75 mL of MBL spiked with the grown culture, and kept with no aeration under the incubation conditions described above for the main cultures (Figure 2).

Figure 2 – Inocula of *C. vulgaris* and *R. subcapitata*.

2.2.2.2. *Lemna minor* and *Lemna gibba*

Cultures of *L. minor* and *L. gibba* were maintained in 500 mL Erlenmeyers filled with ca. 200 mL of Steinberg medium (OECD, 2006a), at 20°C with a photoperiod of $16h^{Light}$:$8h^{Dark}$ (light intensity: ≈2000 LUX), under sterile conditions (Figure 3). The Erlenmeyers and the medium were sterilized by autoclaving (60-90 min, 120°C, 1 atm). Cultures were renewed once a week under approximately sterile conditions (close to the flame).

Figure 3 - Culture of *L. minor* (on the left) and *L. gibba* (on the right).

2.2.3. Bioassays

2.2.3.1. <u>Growth inhibition tests with microalgae</u>

Growth inhibition tests with *R. subcapitata* and *C. vulgaris* followed the recommendations of the OECD guideline 201 (OECD, 2011). The protocol was adapted to the use of 24-well microplates (Geis et al., 2000). Tests started with algae cultures standing in the exponential

growth phase (generally 4 days-old). These cultures were established in a dedicated 75 mL inoculum prepared as indicated above, this constituting a parallel step to the culturing routine. The inoculum cell density was measured using a Neubauer's haemocytometer; then, cell density in the inoculum was adjusted to 10^5 cells/mL by dilution with MBL, so that the cell density at the beginning of the tests could be of 10^4 cells/mL considering the dilution involved in the test set up (see below). Test medium (MBL) and all labware used to prepare the assays (beakers, flasks, etc.) were cleared of contaminants by autoclave sterilization after dedicated decontaminating washing, and microplates were disposable. Test setup was made under approximately sterile conditions (close to flame).

R. subcapitata and *C. vulgaris* were exposed to geometric concentration ranges of nicosulfuron, terbuthylazine, Winner Top® and a customized mixture of the two a.i.s respecting the formulation ratio (i.e. mimicking the Winner Top® before any addition of formulants) (Table 2). The exposure was run in 24-well microplates containing 1 mL of test volume per well, following the scheme exposed in Figure 4. Blank MBL medium was used as the negative control. Three replicates were used per concentration. Separate experiments were conducted with the commercial formulation, the customized mixture of the active ingredients and each active ingredient.

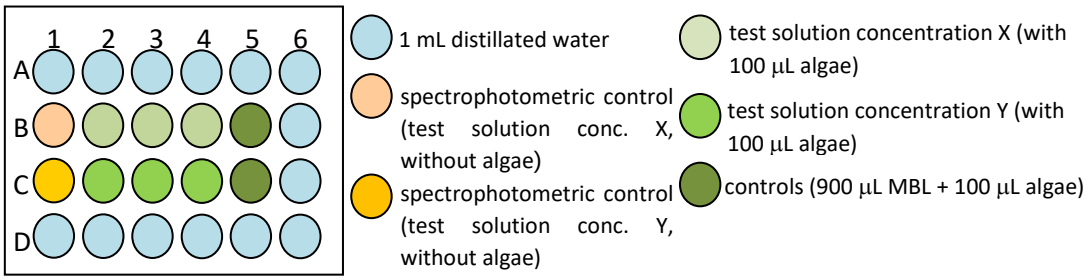


Figure 4 – Scheme representing a filled 24-well microplate with two tested concentrations, X and Y. Only lines B and C and columns 2-5 hold experimental treatments; spectrophotometric controls (reading blanks) are used to prevent a possible interference of the test solution in the absorbance readings, which was not the case here since none test solution was colored. The lines A and D and column 6 are filled with distillated water to minimize the evaporation during the test period.

Table 2 - Concentrations of nicosulfuron (Nic.) and/or terbuthylazine (Terb.) used in exposures of the microalgae species *Raphidocelis subcapitat*a and *Chlorella vulgaris* to each a.i. (nicosulfuron and terbutilazine), to Winner Top® (W.T.) and to the customized mixture of the two a.i. respecting the Winner Top® formulation ratio (f.r.). A schematic representation of the formulant concentration gradient throughout the range of concentrations established using Winner Top® was added for clarity purposes.

Species	Toxic		Concentrations (µg/L)										
Raphidocelis subcapitata		Nic.	0; 3000; 3840; 4915; 6291; 8053; 10308; 13194; 16888; 21617; 27670; 35418; 45335; 58028										
		Terb.	0; 15; 20; 25; 33; 43; 56; 72; 94; 122; 159; 207; 269										
	W.T.	Terb.	0	10	14	20	27	38	54	75	105	148	207
		Nic.	0	0.7	0.9	1.3	1.8	2.5	3.6	5.0	7.0	9.9	13.9
		Formulants											
	f.r.	Terb.	0	5	7	10	14	19	27	38	53		
		Nic.	0	0.3	0.5	0.7	0.9	1.3	1.8	2.5	3.6		
Chlorella vulgaris		Nic.	0; 600; 810; 1094; 1476; 1993; 2690; 3632; 4903; 6619										
		Terb.	0; 15; 20; 25; 33; 56; 72; 94; 122; 159; 207; 269										
	W.T.	Terb.	0	372	483	628	817	1062	1380	1795	2333	3033	
		Nic.	0	24.9	32.4	42.1	54.7	71.2	92.5	120.3	156.3	203.2	
		Formulants											
	f.r.	Terb.	0	75	105	147	206	288	403	565	791	1107	
		Nic.	0	5.0	7.0	9.8	13.8	19.3	27.0	37.9	53.0	74.2	

Tests were incubated at 23°C under continuous light (intensity: ≈6400 LUX) in a climatic chamber, which allowed unrestricted exponential growth under nutrient-sufficient conditions and in blank medium during a period of 96 hours (Figure 5). To prevent cell clumping and promote gas exchange, the algal suspension in each well was thoroughly mixed by repetitive pipetting twice a day (close to the flame). At the end of the exposure period, optical density at 440 nm (UV-Vis Spectrophotometer; Shimadzu UV-1800) was determined for all replicates (OECD, 2011). Optical density values were then converted to cell density records on the basis of a previously established calibration curve ($R^2 = 0.9893$):

$$\mathbf{C = 6931 + [(23179166 \times OD) - (9972459 * OD^2)]}$$

where C is the algae concentration (cells/mL) and OD is the optical density obtained at a wavelength of 440 nm.

The microalgae production in each individual treatment (yield) was calculated as the difference between the algae concentration at the end and the beginning of the test. The inhibition in yield (I_Y) was then expressed as:

$$\boldsymbol{Iy} = \frac{(\boldsymbol{Yc} - \boldsymbol{Yt})}{(\mathbf{Yc} \times \mathbf{100})}$$

where Y_C and Y_T represent the mean value of yield for the controls and the yield in each replicated treatment, respectively.

Figure 5 – Growth inhibition test with *R. subcapitata* and *C. vulgaris.* Microplates were randomly distributed in the climatic chamber and they were daily moved around to prevent any effect of any unexpected spatial variation in the incubation conditions.

2.2.3.2. Growth inhibition tests with macrophytes

Growth inhibition tests with *L. minor* and *L. gibba* followed the recommendations of the OECD guideline 221 (OECD, 2006a). Test medium (Steinberg medium) and all equipment used for the tests (beakers, flasks, etc.) were clear of chemical contaminants by dedicated washing followed by autoclave sterilization.

Tests were carried out in disposable 6-well macroplates (final volume per well: 10 mL) as adapted by Kaza et al. (2007) and Kolasińska et al. (2010), at 23°C, under continuous light (intensity: ≈1700 LUX), during 7 days (Figure 6). Tests started by inoculating each well with 3 healthy colonies with 3 fronds each, which were allowed to grow as monocultures exposed to concentrations ranges of nicosulfuron, terbuthylazine, Winner Top® and the corresponding customized mixture of the a.i.s (Table 3). The test design included 3 replicates at each test concentration and 6 control replicates. Three extra replicates were collected from the culture for determination of the average dry weight until constant weight (normally 24 h at 60°C) at the beginning of the test. The macroplates were moved once a day in the climatic chamber to prevent the effect of non-homogeneous incubation conditions. After 7 days of exposure under these conditions, all colonies were collected from each replicate, fronds were counted, then rinsed with distillated water and blotted in absorbent paper to remove excess water (the root fragments were also included). They were then dry at 60°C until constant weight (normally during 24 hours) for the determination of final dry weight. *Lemna* biomass (yield) for each individual treatment was calculated, considering either frond number or dry weight, as the difference between records at the end and at the beginning of the test. Yield inhibition was calculated as described above for microalgae (see section 2.2.3.1.)

Figure 6 - Growth inhibition test with *L. gibba.* Macroplates were randomly distributed in the climatic chamber.

Table 3 - Concentrations of nicosulfuron (Nic.) and/or terbuthylazine (Terb.) used in exposures of the macrophytes *Lemna minor* and *Lemna gibba* to each a.i. (nicosulfuron and terbutilazine), to Winner Top® (W.T.) and to the customized mixture of the two a.i.s respecting the Winner Top® formulation ratio (f.r.). A schematic representation of the formulant concentration gradient throughout the range of concentrations established using Winner Top® was added for clarity purposes.

Specie	Toxic		Concentrations (µg/L)										
Lemna minor		Nic.	0; 1; 2; 3; 4; 7; 10; 17; 27; 43; 69										
		Terb.	0; 20; 33; 52; 84; 134; 215; 344; 550										
	W.T.	Terb.	0	50	70	98	137	192	269	376	527	738	1033
		Nic.	0	3.4	4.7	6.6	9.2	12.9	18.0	25.2	35.3	49.4	69.2
		Formulants											
	f.r.	Terb.	0	10	16	26	41	66	105	168	268	429	687
		Nic.	0	0.7	1.1	1.7	2.7	4.4	7.0	11.3	18.0	28.7	46.0
Lemna gibba		Nic.	0; 1; 2; 3; 4; 7; 10; 17; 27; 43; 69										
		Terb.	0; 8; 13; 20; 33; 52; 84; 134; 215; 344; 550										
	W.T.	Terb.	0	50	70	98	137	192	269	376	527	738	1033
		Nic.	0	3.4	4.7	6.6	9.2	12.9	18.0	25.2	35.3	49.4	69.2
		Formulants											
	f.r.	Terb.	0	10	16	26	41	66	105	168	268	429	687
		Nic.	0	0.7	1.1	1.7	2.7	4.4	7.0	11.3	18.0	28.7	46.0

2.2.4. Data analysis

EC_{50} values and respective 95% confidence intervals corresponding to data retrieved in bioassays with microalgae and *Lemna* spp. (continuous variables - yield inhibition records) were estimated by non-linear regression, using the least-squares method to fit the data to the logistic equation; calculations were made in Statistica 8 (Statsoft) (E.C., 2007). Because EC_{10} and EC_{20} values are standard benchmarks in the environmental risk assessment of chemicals, including PPPs (E.C.B., 2003), they were also estimated through the same method as used for the EC_{50} estimation. Similarly, Lowest Observable Effect Concentrations (LOECs) were determined by applying a one-way ANOVA followed by the post-hoc Dunnet test ($p<0.05$) to each test outcome.

In order to facilitate the comparison between tests with commercial formulation and the corresponding customized mixture of a.i.s, concentration ranges used were additionally transformed into dimensionless, hence fully comparable Toxic Unit (TU) ranges. The sum of the quotients $Ci/EC_{50}i$ was applied for the purpose, considering i^{th} components of the mixture (in this case, nicosulfuron and terbuthylazine), and assuming that C is the concentration of *i* within the mixture and EC_{50} is the median effect concentration found in single-chemical exposures to *i* (see Jonker et al., 2005 for more details on the TU approach).

2.3. RESULTS

The graphs representing the relationship between the tested toxic and the respective inhibition in yield (%) for each test outcome are presented together for each tested microalgae (Figure 7 and 8) and macrophyte (Figure 9 and 10) species. In all cases (species x toxicant challenge), a significant impairment of the assessed parameter was found (Table 4), meaning that both the a.i.s and the formulations are able to significantly depress the growth of all species. The estimated concentrations of the a.i.s individually and in formulation that induce a negative effect of 10, 20 and 50% in the endpoint assessed (EC_{10}, EC_{20} and EC_{50}) are presented in Table 5.

Table 4 – One-way ANOVA summary (df, degrees of freedom) regarding the response (biomass yield) of *R. subcapitata, Chlorella vulgaris, Lemna minor and Lemna gibba* following exposure to nicosulfuron (Nic.), terbuthylazine (Terb.), Winner Top® (W.T.) and the customized mixture of a.i.s respecting their ratio in the commercial formulation (Nic. + Terb. (f.r.)). LOEC values are presented in µg/L for single exposures to the a.i.s. For exposures to Winner Top® and the mixtures of a.i.s, LOEC values are given in TU, although corresponding concentrations of terbuthylazine (T) and nicosulfuron (N) are also presented within brackets for clarity.

Source of variation	df	$MS_{residual}$	*F*	*P*	LOEC
Raphidocelis subcapitata					
Nic.	13, 37	1.19E+13	44.39	<0.001	3000 µg/L
Terb.	12, 33	3.83E+13	199.20	<0.001	15 µg/L
W.T.	10, 29	2.90E+13	11.77	<0.001	3.62 TU (54 T + 3.62 N µg/L)
Nic. + Terb. (f.r.)	8, 23	3.95E+13	79.58	<0.001	0.47 TU (7 T + 0.47 N µg/L)
Chlorella vulgaris					
Nic.	9, 33	5.98E+13	33.11	<0.001	810 µg/L
Terb.	11, 32	4.07E+13	199.93	<0.001	15 µg/L
W.T.	9, 25	1.42E+13	13.81	<0.001	2.75 TU (483 T + 32.36 N µg/L)
Nic. + Terb. (f.r.)	6, 27	2.82E+13	35.26	<0.001	0.84 TU (147 T + 9.8 N µg/L)
Lemna minor					
Nic.	10, 25	286.63	71.66	<0.001	2 µg/L
Terb.	8,21	430.87	78.45	<0.001	20 µg/L
W.T.	10, 25	233.61	41.27	<0.001	2.22 TU (70 T + 4.69 N µg/L)
Nic. + Terb. (f.r.)	10, 25	359.24	105.04	<0.001	0.82 TU (26 T + 1.74 N µg/L)
Lemna gibba					
Nic.	10, 24	290.07	49.32	<0.001	4 µg/L
Terb.	10, 25	251.63	117.95	<0.001	13 µg/L
W.T.	9, 23	138.92	43.97	<0.001	2.91 TU (50 T + 3.35 N µg/L)
Nic. + Terb. (f.r.)	10, 25	190.23	20.10	<0.001	3.85 TU (66 T + 4.42 N µg/L)

Terbuthylazine was more toxic than nicosulfuron by more than two orders of magnitude to *R. subcapitata*, as visually evident when comparing concentration response curves in Figure 7 A and B. This trend was confirmed by confronting LOEC values of 15 and 3000 µg/L, respectively (Table 4) and estimated EC_x values for terbuthylazine lower by 2-3 orders of magnitude than those for nicosulfuron (Table 5). Graphs C and D in Figure 7 suggest that the mixture of the a.i.s in formulation ratio was more toxic to the species than the commercial formulation, with 70-80 % yield inhibition expected at 2-3 TU compared to 6-8 TU, respectively. This trend was confirmed by the lower LOEC value found for the mixture of a.i.s (Table 4), and validated by distinct EC*x* values, whose confidence intervals generally do not overlap (Table 5). The response of the other microalgae species, *C. vulgaris,* to the different toxicant challenges was very similar. Higher sensitivity to terbuthylazine compared to nicosulfuron was also recorded (Figure 8), although the difference in EC_x values was of lower magnitude but still effective through non-overlapping confidence intervals of the estimates (Table 5). As well as *R. subcapitata*, *C. vulgaris* was more sensitive to the customized mixture of a.i.s compared to the commercial formulation Winner Top®, with a full distinction only recognized at the EC_{50} level by non-overlapping confidence intervals (Figure 8; Table 5).

Comparing the responses of the two algal species (Figures 7 and 8), *C. vulgaris* was the most sensitive to nicosulfuron with a LOEC value of 810 µg/L compared to a LOEC value of 3000 µg/L found for *R. subcapitata*; distinct EC_{50} estimates with no overlapping 95% confidence intervals confirm this interpretation (Table 5). On the contrary, *R. subcapitata* was the most sensitive to terbuthylazine. Despite the similar LOEC values found for the two species (15 µg/L), this pattern was evidenced by the shape of the curves in Figure 7B compared to Figure 8B and validated by distinct EC_{50} estimates with non-overlapping 95% confidence intervals. The comparative sensitivity of the microalgae to Winner Top® was somewhat inconsistent. Based on LOEC values, which are dependent on the concentration ranges tested, *R. subcapitata* should be highlighted as slightly more tolerant than *C. vulgaris* (Table 4), but the order was reversed when accounting to estimated EC_{50} values (Table 5). These denote *R. subcapitata* as significantly more sensitive to Winner Top® with an EC_{50} value of 3.67 TU, compared to the EC_{50} value of 6.84 TU obtained for *C. vulgaris*. Although *C. vulgaris* then presented lower EC_{10} and EC_{20} values, their confidence intervals overlap or the estimate was not statistically significant. *R. subcapitata* was also more sensitive than *C. vulgaris* to the customized mixture of the a.i.s as retrieved from the LOEC values found (Table 4) and by comparing the EC_{50} values of 1.27 and 2.23 TU, respectively (Table 5); a similar trend was found regarding EC_{10} and EC_{20} estimates but overlapping confidence intervals prevented the assumption of a significant difference between species in sensitivity.

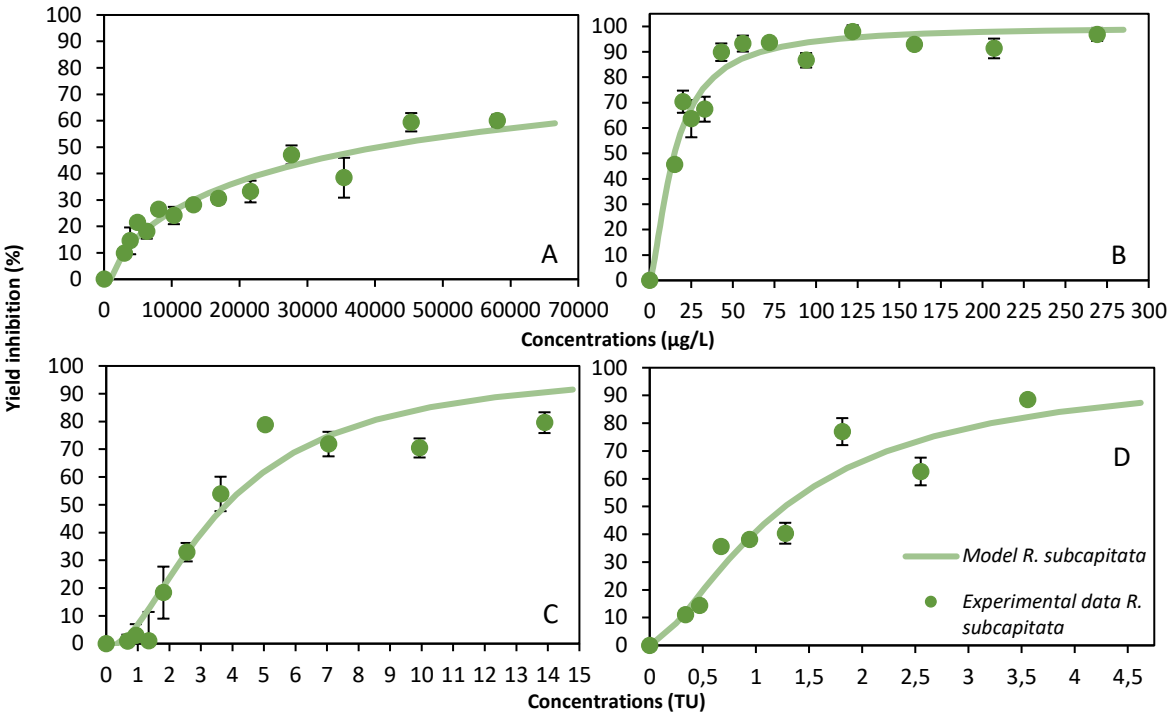


Figure 7 - Inhibition in yield found following exposure (96h) of *Raphidocelis subcapitata* to increasing concentrations of nicosulfuron and terbuthylazine (A and B, respectively) in µg/L; Winner Top® and the customized mixture of a.i.s (C and D, respectively) in TUs. Error bars stand for the standard error. The line added represents the non-linear regression model that best fitted the experimental data for further calculation of the EC_x values (Table 5).

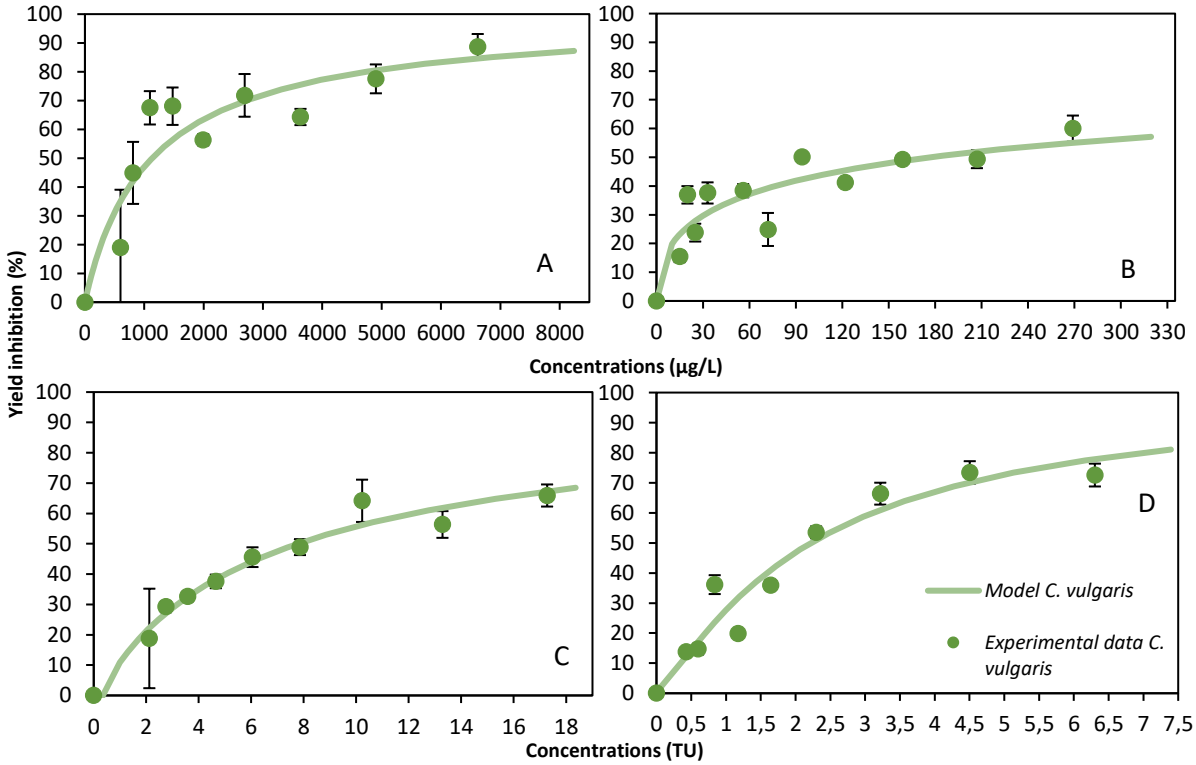


Figure 8 – Inhibition in yield found following exposure (96h) of *Chlorella vulgaris* to increasing concentrations of nicosulfuron and terbuthylazine (A and B, respectively) in µg/L; Winner Top® and the customized mixture of a.i.s (C and D, respectively) in TUs. Error bars stand for the standard error. The line added represents the non-linear regression model that best fitted the experimental data for further calculation of the EC_x values (Table 5).

A direct graphical interpretation shows terbuthylazine as the least toxic a.i. *to L. minor* (Figure 9 A,B), which contrasts to the results obtained following microalgae exposure (see above). This trend was confirmed by LOEC values (Table 4) and statistically distinct EC_x estimates whose confidence intervals do not overlap (Table 5). Similarly to the outcome of microalgae testing, the mixture of a.i.s was more toxic than the commercial formulation to the *L. minor* (Figure 9 C,D), as confirmed by LOEC values of 0.82 and 2.22 TU, respectively (Table 4) and increasingly distinct EC_x estimates (e.g. EC_{50} estimates of 1.62 TU and 4.70 TU, respectively, with non- overlapping confidence limits; Table 5).

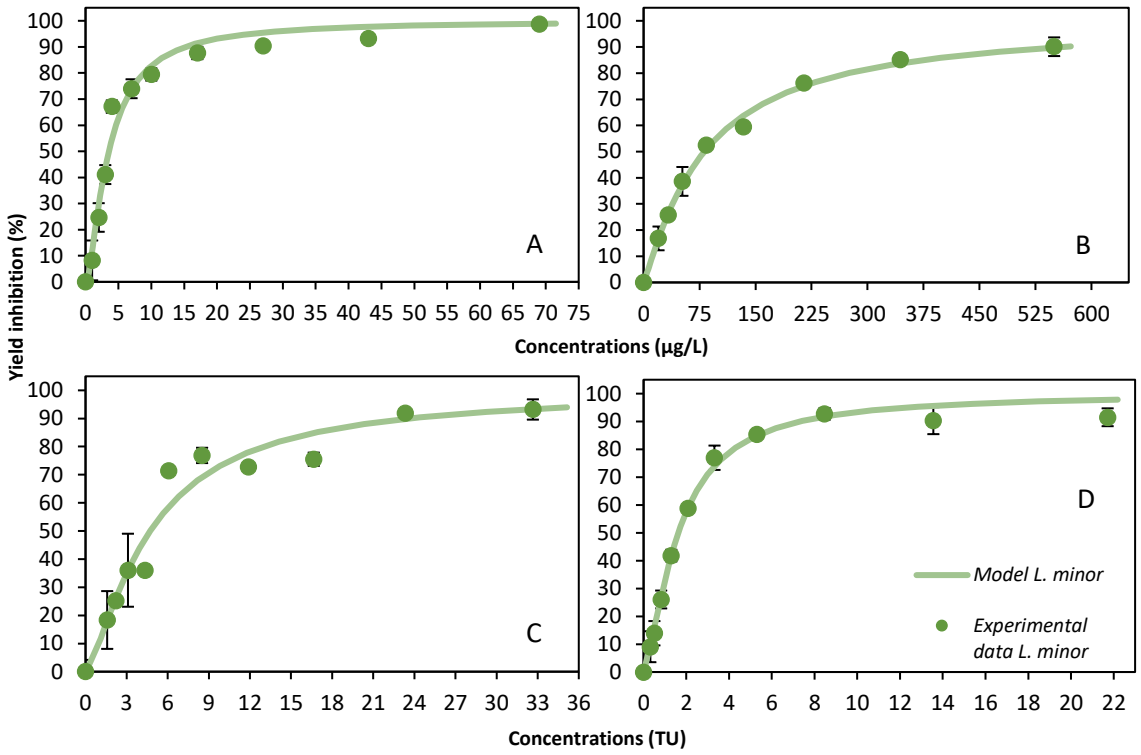


Figure 9 – Inhibition in yield found following exposure (7 days) of *Lemna minor* to increasing concentrations of nicosulfuron and terbuthylazine (A and B, respectively) in µg/L; Winner Top® and the customized mixture of a.i.s (C and D, respectively) in TUs. Error bars stand for the standard error. The line added represents the non-linear regression model that best fitted the experimental data for further calculation of the EC_x values (Table 5).

L. gibba also showed a higher sensitivity to nicosulfuron (Figure 10 A,B), with a LOEC value of 4 µg/L compared to 13 µg/L found for terbuthylazine (Table 4); consistently lower, distinct EC_x estimates confirmed the trend shown by LOEC values (Table 5). However, unlike all the other species, *L. gibba* was more sensitive to Winner Top® than to the customized mixture of a.i.s (Figure 10 C,D), as indicated by LOEC values of 2.91 and 3.85 TU, respectively (Table 4); this difference in sensitivity was not validated by EC_x estimation, since for all equi-effective benchmarks, overlapping confidence intervals were found when comparing between formulations (Table 5).

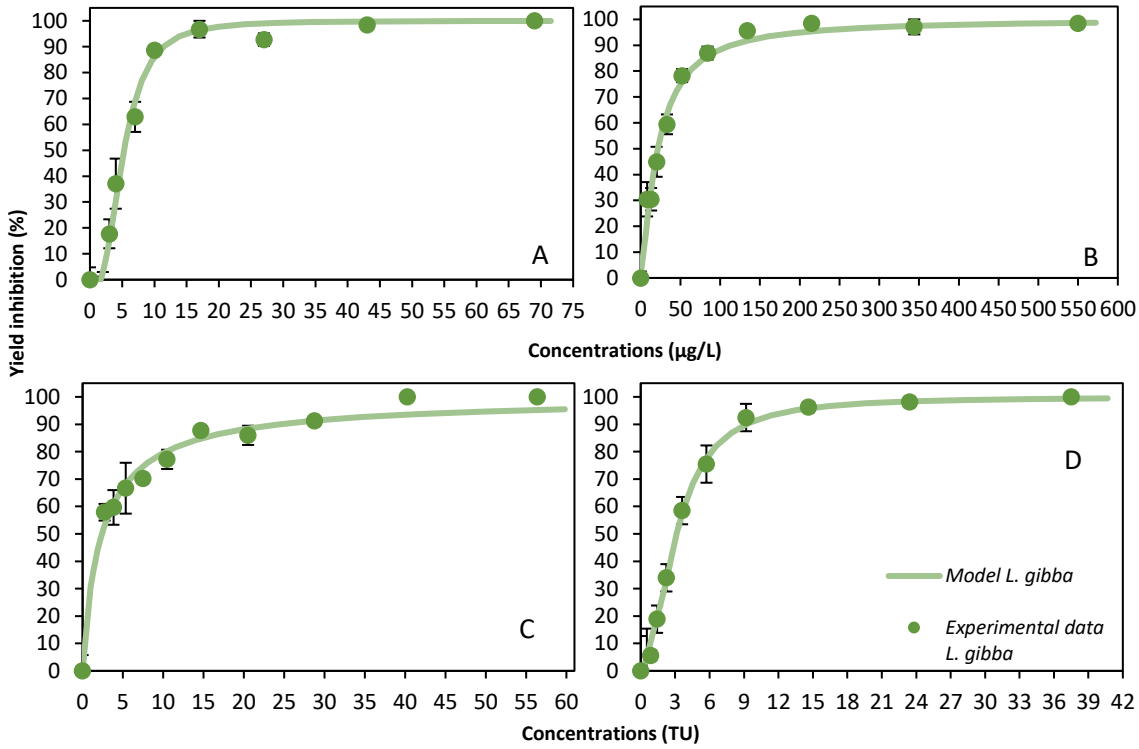


Figure 10 – Inhibition in yield found following exposure (7 days) of *Lemna gibba* to increasing concentrations of nicosulfuron and terbuthylazine (A and B, respectively) in µg/L; Winner Top® and the customized mixture of a.i.s (C and D, respectively) in TUs. Error bars stand for the standard error. The line added represents the non-linear regression model that best fitted the experimental data for further calculation of the EC_x values (Table 5).

The response of the two species of macrophytes to nicosulfuron was apparently very similar, as interpreted from panels A in Figures 9 and 10, and by very close LOEC values (Table 4). This similarity in sensitivity to nicosulfuron was not confirmed by comparing between distinct, non-overlapping EC_x estimates for both species. On the other hand, *L. gibba* was the macrophyte species most sensitive to terbuthylazine (panels B in Figures 9 and 10; Tables 4, 5). Regarding the response of the two macrophyte species to the customized mixture of a.i.s, *L. minor* was clearly more sensitive than *L. gibba* showing LOEC values of 0.82 and 3.85 TU, respectively (panel D in Figures 9 and 10; Table 4), and generally non-overlapping EC_x estimates (Table 5). In contrast, *L. gibba* was the most sensitive species to Winner Top®, with close LOEC values (Table 4) but non-overlapping EC_{50} and EC_{20} values of 2.57 and 0.60 TU, respectively (Table 5).

Table 5 - Estimated concentrations of the a.i.(s), individually and in formulation, that induce 50, 20 and 10 % reduction (EC_{50}, EC_{20} and EC_{10}) in the biomass yield of the tested species, with the respective 95% confidence intervals within brackets. These values were estimated by non-linear regression, using the least-squares method to fit the data to the logistic equation. EC_x values are presented in µg/L for single chemical exposures and in Toxic Units (TU) for the formulation exposures with the respective conversion to µg/L for both a.i.s.

		Single chemical exposures		Formulation exposures	
		Nic. (µg/L)	**Terb. (µg/L)**	**W.T.**	**Nic + Terb (f.r.)**
R. subcapitata	EC_{50}	**36209** (28242-44177)	**14.92** (12.28-17.56)	**3.67 TU** (2.61-4.73) 54.69 T + 3.67 N µg/L	**1.27 TU** (1.05-1.48) 18.89 T + 1.27 N µg/L
	EC_{20}	**4806** (3017-6596)	**5.79** (3.65-7.93)	**1.64 TU** (0.82-2.47) 24.61 T + 1.64 N µg/L	**0.50 TU** (0.35-0.66) 7.48 T + 0.50 N µg/L
	EC_{10}	**1473** (623-2322)	**3.33** (1.68-4.97)	**1.03 TU** (0.33-1.72) 15.32 T + 1.03 N µg/L	**0.30 TU** (0.17-0.42) 4.34 T + 0.29 N µg/L
C. vulgaris	EC_{50}	**1122.76** (687.15-1558.38)	**177.49** (85.40-269.58)	**6.84 TU** (4.72-8.96) 1201.34 T + 80.48 N µg/L	**2.23 TU** (1.77-2.70) 391.65 T + 25.82 N µg/L
	EC_{20}	**267.20** (24.66-509.74)	**10.26*** (0-20.62)	**1.39 TU** (0.42-2.36) 243.49 T + 16.31 N µg/L	**0.71 TU** (0.44-0.98) 124.78 T + 8.36 N µg/L
	EC_{10}	**115.26*** (0-262.62)	**1.93*** (0-5.07)	**0.54 TU*** (0-1.11) 95.61 T + 6.41 N µg/L	**0.36 TU** (0.17-0.56) 63.72 T + 4.27 N µg/L
L. minor	EC_{50}	**3.46** (2.84-4.08)	**81.29** (67.62-95.00)	**4.70 TU** (3.66-5.74) 148.39 T + 9.94 N µg/L	**1.62 TU** (1.39-1.85) 51.15 T + 3.43 N µg/L
	EC_{20}	**1.37** (0.94-1.80)	**24.22** (17.04-31.40)	**1.70 TU** (1.04-2.36) 53.76 T + 3.60 N µg/L	**0.63 TU** (0.48-0.77) 19.74 T + 1.32 N µg/L
	EC_{10}	**0.79** (0.46-1.14)	**11.88** (7.14-16.63)	**0.94 TU** (0.46-1.42) 29.67 T + 1.99 N µg/L	**0.36 TU** (0.24-0.47) 11.31 T + 0.76 N µg/L
L. gibba	EC_{50}	**5.16** (4.36-5.95)	**22.07** (18.61-25.53)	**2.57 TU** (1.65-3.50) 44.14 T + 2.96 N µg/L	**3.24 TU** (2.41-4.08) 55.60 T + 3.73 N µg/L
	EC_{20}	**3.15** (2.38-3.92)	**7.78** (5.67-9.89)	**0.60 TU** (0.15-1.04) 10.25 T + 0.69 N µg/L	**1.63 TU** (0.91-2.35) 27.97 T + 1.87 N µg/L
	EC_{10}	**2.36** (1.59-3.13)	**4.23** (2.69-5.76)	**0.25 TU** (0.15-1.04) 4.36 T + 0.29 N µg/L	**1.09 TU** (0.45-1.73) 18.70 T + 1.25 N µg/L

* Non-significant estimation

2.4. DISCUSSION

As a general outcome, this study showed that Winner Top®, the mixture of its a.i.s respecting the formulation ratio, and its a.i.s nicosulfuron and terbuthylazine *per se*, can significantly affect the growth of all tested species at the tested concentrations. Some published studies report the occurrence of nicosulfuron and terbuthylazine in the aquatic environment, particularly in surface waters, and the integration of these records with our ecotoxicological outcome provides the necessary grounds for a more realistic discussion on the actual hazardous potential of the pesticides.

Azevedo et al. (2000) detected terbuthylazine concentrations of 0.02 and 1.65 µg/L in surface water samples collected in different reservoirs in Portugal, namely in "Porto de Carvoeira" and in "Albufeira de Póvoa e Meadas", respectively. In the Spring of 2007, Palma et al. (2009) found a terbuthylazine maximum concentration of 112.4 ng/L in a surface water sample collected in the Alqueva reservoir, in the South of Portugal. A more recent study by Palma et al. (2014) noticed an increase of terbuthylazine concentration (532 ng/L) in the same sampling site. In this study, terbuthylazine was the only compound detected in all water samples and it was one of the pesticides present in higher concentrations throughout the Alqueva reservoir. A maximum terbuthylazine concentration of 0.24 µg/L was also found in the river Ebro basin, in a study involving the collection of several surface and groundwater samples in three different river basins (Hildebrandt et al., 2008). These and other studies (e.g. Postigo et al., 2010; Sass and Colangelo, 2006) seem to indicate that the levels of terbuthylazine in surface waters have been increasing, which can relate to the ban of atrazine in Europe requiring an adequate replacer for the control of agricultural weeds. In spite of this raise, terbuthylazine concentrations found in surface waters are so far below the concentrations that cause a significant deleterious effect on the model primary producers tested in the present study. The highest environmental concentration found was of 1.65 µg/L (Azevedo et al. 2000) while the lowest benchmark noticing negative impacts (LOEC or EC_{20}; E.C.B., 2003) was the EC_{20} value of 5.79 µg/L, thus within the same order of magnitude, determined in the present study for *R. subcapitata*. The highlighted environmental concentration is not too far from the lower benchmark we found, and almost twenty years have passed since the records by Azevedo et al. (2000). Assuming the increasing trend for terbuthylazine levels in surface waters as discussed above, and considering that long-term exposure to sequential inflows of the contaminant may translate into more pronounced effects in the aquatic biota, it is reasonable to raise the concern on the real hazardous potential of terbuthylazine at least for primary producers and consequently to freshwater ecosystems.

Considering the other active ingredient, nicosulfuron, there is a gap in literature regarding the assessment of its occurrence in surface waters. Gonzalez-Rey et al. (2015) evaluated the occurrence of pharmaceutical compounds, including nicosulfuron, in Arade River estuary (Portugal) but the concentrations of this a.i. in the collected samples were below the levels of detection. Battaglin et al. (2000) assessed the occurrence of sulfonylurea and other herbicides in rivers, reservoirs and ground water in the Midwestern

United States in 1998 and they found a maximum nicosulfuron concentration of 0.266 µg/L in water samples from Midwestern streams and rivers. Struger et al. (2011) assessed the occurrence and distribution of sulfonylurea and related herbicides in Central Canadian surface waters between 2006 and 2008 and the maximum concentration of nicosulfuron found was of 525 ng/L in 2006. Similar to terbuthylazine, the maximum environmental concentration found so far (0.266 µg/L; Battaglin et al. 2000) is below but not too far from the lowest LOEC and EC_{20} values determined in our study (2 µg/L and 1.37 µg/L to *Lemna minor,* respectively). Further studies are required for a wide update of actual concentrations of nicosulfuron in surface waters, this allowing a more comprehensive view on the hazardous environmental potential of nicosulfuron. In fact, the environmental risk assessment of this pesticide in Europe exposed its very high toxicity to macrophyte species, thereby constraining the pesticide use to the establishment of a mandatory buffer zone between application sites and adjacent waterways (EFSA, 2007). Such a recommendation denotes a marked concern on the suitability of nicosulfuron to promote adverse environmental effects in surface water ecosystems by affecting primary producers.

The range of microalgae sensitivity to terbuthylazine was very wide, with equi-effective concentrations differing by up to one order of magnitude between the most sensitive *R. subcapitata* and the most tolerant *C. vulgaris*. Differential sensitivity to terbuthylazine was also found for macrophytes, with *L. gibba* being significantly more sensitive than *L. minor*. Amongst the species tested, the microalgae *R. subcapitata* was the most sensitive to this chemical hence the one delivering the most environmentally protective benchmarks. Our results were consistent with the literature, where the high sensitivity of microalgae to triazines was also demonstrated (Fairchild et al., 1997; Ma et al., 2006; Pérez et al., 2011). For example, Pérez et al. (2011) found a terbuthylazine 72 h-EC_{50} of 24 µg/L for *R. subcapitata* growth, and Sbrili et al. (2005) determined an even lower 72 h-EC_{50} (9 µg/L) than that obtained in our study for *R. subcapitata* growth. Cedergreen & Streibig (2005) found that *R. subcapitata* was more sensitive to terbuthylazine than the macrophyte specie *L. minor*, which are records consistent with our own. Munkegaard et al. (2008) recorded an estimated terbuthylazine 7 d-EC_{50} value of 157 µg/L for *L. minor*, which is almost twice the value obtained in our study. However, the parameter evaluated in this case was the growth rate, whose magnitude is buffered by normalising to the logarithmic time range of the test thus possibly explaining a higher responsiveness of yield inhibition as used in our study.

On the other hand, the microalgae *C. vulgaris* was more sensitive than *R. subcapitata* to nicosulfuron, both showing equi-effective concentrations more than three orders above their terbuthylazine counterparts. Ma et al. (2002) determined a nicosulfuron 96 h-EC_{50} value of almost an order of magnitude below ours for *C. vulgaris*, while a nicosulfuron 96 h-EC_{50} value very close to that determined in our study was recorded later to *R. subcapitata* (Ma et al. 2006). The macrophytes *L. minor* and *L. gibba* showed similar sensitivity to the pesticide, with *L. minor* being the most sensitive species among all species tested. Mohammad et al. (2005) found a nicosulfuron 7 days-EC_{50} value of 14.5 µg/L for *Lemna sp.* growth and Lewis et al. (2016) found a nicosulfuron 7 days-EC_{50} value of 2 µg/L for *L. gibba* biomass. The noticed difference in sensitivity between microalgae and

macrophytes is supported by the literature. Fairchild et al. (1997) compared the sensitivity of *R. subcapitata* and *L. minor* to sixteen herbicides, including two sulfonylureas; for both sulfonylureas, *L. minor* was more sensitive than the microalgae by four levels of magnitude. Munkegaard et al. (2008) also showed that *L. minor* was more sensitive than *R. subcapitata* to a sulfonylurea herbicide, metsulfuron-methyl, by estimating 7 d- and 48 h-EC_{50} values of 0.51 and 1934 µg/L, respectively. Cedergreen & Streibig (2005) confirmed the trend for higher sensitivity of *L. minor* to metsulfuron-methyl and to another sulfonylurea (triasulfuron).

It is worth remarking that macrophytes were much more sensitive to nicosulfuron while microalgae were much more sensitive to terbuthylazine, which reveals an opposite trend within producers. This difference in sensitivity to each herbicide, as well as the inversion in sensitivity order between macrophytes and microalgae can relate to several physical, chemical and biological features involved in the test systems. First, the amount of chemical that reached the physiological site of action may have been distinct given that distinct absorption pathways are placed when comparing microalgae (contact absorption only) and rooted macrophytes (systemic and contact absorption). Linked with absorption pathways is the octanol-water partition coefficient (Kow) of the tested chemicals. The Kow of terbuthylazine is much higher than that of nicosulfuron (see Table 1), meaning that the former compound has higher solubility in lipids. Lipid-soluble substances easily enter cells through the cell membranes (Reddy and Locke, 1996), i.e. terbuthylazine can more easily enter cells via contact absorption than nicosulfuron. In this way, surface-contact should be a major terbuthylazine uptake pathway both in microalgae and macrophytes, which should contribute to a lower distance in sensitivity between the organisms compared to the poorly lipid-soluble nicosulfuron. Consistently, high sensitivity of microalgae was already reported for other systemic herbicides, which may be ruled by the rationale above (see e.g. Bražėnaitė et al. 2006 for the toxicity of pendimethalin, with a high Kow of 5.4, to *R. subcapitata*). Also, a parallel can be made with the results by Rioboo et al. (2002), which agree with ours. In their study, *C. vulgaris* was exposed to a phenylurea (such as nicosulfuron) and a triazine (such as terbuthylazine), and growth inhibition was also higher following exposure to the triazine. The Kow of the triazine was also higher than the Kow of the phenylurea, although the difference was not as pronounced as in our study. The converse evidences regarding herbicides touted similarly as of systemic action show that care must be taken in the selection of the most appropriate non-target organism depending on the specific aims of each study, with physico-chemical properties and intake routes of pesticides being key intertwining properties featuring the expected outcomes.

Also noteworthy is the distance between the toxicity response of the two macrophyte species (belonging to the same genus) or the two microalgae species. *R. subcapitata* and *L. gibba* were markedly more sensitive to terbuthylazine than their counterparts. Regarding the exposure to nicosulfuron, *C. vulgaris* and *L. minor* were the most sensitive test organisms but the distance in sensitivity ranges was not as marked as noticed for terbuthylazine. In fact, such a species-dependent variation within similar organisms responding to a same herbicide is common. Ferraz et al. (2004) studied the effects of propanil in the growth of four green microalgal species and found distinct 72 h-EC_{50} values by more than one order of magnitude. Vidal et al. (2011) exposed different green unicellular microalgae species, including *C. vulgaris* and *R. subcapitata*, to

phenmedipham, and found 96 h-EC_{50} values for growth which were distinct by more than one order of magnitude. Cedergreen & Streibig (2005) determined a pendimethalin 7 days-EC_{50} of 0.634 mg/L for *Lemna minor* while Turgut & Fomin (2002) determined much higher pendimethalin 14 days-EC_{50} values (10.74-24.13 mg/L) for another rooted macrophyte, *Myriophyllum aquaticum*. The interplay between the herbicides lipophilicity and the organism's surface-to-volume ratio may play a role in explaining these sensitivity variations. The shape and size of *R. subcapitata* cells compared to that by *C. vulgaris* cells is likely to translate into greater surface-to-volume ratios; also, the contact of *L. gibba* with the waterborne toxicant should be greater than that by *L. minor* provided the much larger area of the inner frond surface and much longer and larger roots. Under this rationale, the comparative sensitivity ranges to terbuthylazine found within the present study seem consistent.

There was no clear pattern on whether microalgae or macrophytes were the most sensitive to the formulations tested (Winner Top® and corresponding formulation of the a.i.s terbuthylazine and nicosulfuron), as it dependended mostly on the species rather than on the producer group. In spite of this unexpected outcome - given the systemic nature of the PPP driving to the assumption that *Lemna* sp. would be by far the most sensitive non-target indicator -, *L. minor* was very sensitive to the formulation ratio (similarly to *R. subcapitata*). Based on this evidence and considering the aims and experimental design of the following chapter of this dissertation, this macrophyte was selected for further studies. Furthermore, in all cases but for *Lemna gibba*, the commercial formulation Winner Top® seemed to have a protective effect compared with the customized mixture of the a.i.s respecting the commercial formulation ratio. Distinct toxicity was found between these two formulations, which provides an indirect measure of the contribution of the so-called inert ingredients to the overall toxicity of the product. Therefore, it became clear that the additional ingredients conjugated with the a.i.s seem to have a protective effect by generally reducing the toxicity of the formulation to the tested non-target organisms, this being probably due to interactions between formulants and the a.i.s, amending their bioavailability. Because formulants were found to have a role in modulating the toxicity of the commercial formulation (even though they decreased the toxicity of the a.i.s), their touting as inert ingredients is clearly inadequate.

There are previous studies confirming the non-inert property of formulants within pesticide formulations, although their capacity to increase the toxicity of the formulation was more frequently reported. Cedergreen & Streibig (2005) reported this stimulating role of the formulants using a glyphosate formulation. Nevertheless, these authors tested other herbicides namely terbuthylazine, metsulfuron-methyl, pendimethalin and triasulfuron and respective formulated products with *L. minor* and *R. subcapitata*, and the herbicide formulations did not enhance (nor repressed) herbicide toxicity towards aquatic species. Pereira et al. (2009) also verified that the Spasor® formulation was more toxic than the respective a.i., glyphosate, to the microalgae *R. subcapitata*, and Pereira et al. (2000) compared the toxicity of commercial formulations and water samples fortified with the respective a.i. (e.g. propanil, MCPA, molinate), and in most cases the formulations were significantly more toxic to *Daphnia* and *Thamnocephalus*. Consistently with our results, *R.*

subcapitata showed significantly higher sensitivity to water samples fortified with propanil and molinate compared to the respective commercial formulations in the study by Pereira et al. (2000). All that said confirms the importance of studying the formulations as a whole or at least the combination of their active ingredients. Unlike most published studies, the present one innovatively allowed the comparison of commercial formulation with a version of itself free of formulants, thus allowing a direct view on the true contribution of formulants to the ecotoxicity of the commercial product.

2.5. CONCLUSIONS

Based upon the results of this study, a primary conclusion is that the growth of aquatic primary producers can be significantly affect by Winner Top®, the mixture of its a.i.s in formulation ratio, and its a.i.s nicosulfuron and terbuthylazine *per se.* Such deleterious effects can be expected at concentrations slightly above reported concentrations in natural surface water systems, which raise concerns on the pesticides environmental safety provided the verified trend for the increase of residues in surface waterbodies or the likelihood of a more continuous, long-term exposure of the biota. Great differences in sensitivity to the tested compounds between the different groups of organisms tested and within these groups were noticed, which evidences the importance of judiciously select test organisms to better comply with environmentally precautionary principle in the risk assessment of pesticides. Finally, but not of lessen importance, this study confirmed an active role of the so called-inert ingredient in the overall toxicity of Winner Top® through direct comparison with a version of the commercial formulation where only the active ingredients were combined. In this particular case, the un-known formulants seem to have a protective effect towards the non-target indicators tested, this being contrary to a more typical toxicity enhancement by formulants or adjuvants. The present study hence reinforces the need for further action by the competent authorities towards a more stringent PPP legislation that properly covers un-known ingredients, and their possible interactions with other formulation components, within risk assessment frameworks developed to comply with marketing authorization requests. On the other hand, the agrochemicals industry should be challenged by the generated evidences to continue innovating in the formulation of their products in an attempt to produce eco-friendlier alternatives. Pressure should also be made towards a wider disclosing policy by the agrochemicals industry, so that the feasibility of benchmarks retrieved in environmental risk assessment prior licensing can be improved.

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CHAPTER 3 – Eco-friendlier alternative formulations of the commercial herbicide Winner Top®

3.1. INTRODUCTION

Agriculture relies on the use of various plant protection products (PPPs) to ensure improvements in quality and yield of crops so as to sustain a growing human population (Carlile, 2006; Damalas and Eleftherohorinos, 2011; Grube et al., 2011). However, their excessive use has been bringing some problems to human health and the environment through the impacts they drive on non-target organisms.

The application of PPPs normally takes place in the terrestrial environment but they can reach surface water through different transport pathways, e.g. runoff and leaching, often causing hazardous contamination scenarios (Abrantes et al., 2010; Battaglin et al., 2014; Carriger and Rand, 2008). Surface water contamination by PPPs happens worldwide and has been matter of concern and debate within the scientific community (Albanis and Hela, 1998; Cerejeira et al., 2003; Huber et al., 2000; Konstantinou et al., 2006; Planas et al., 1997). Regulatory agencies worldwide have already recognized this problem and have been developing tighter screening protocols and authorization requirements, as well as comprehensive assessment instruments. Amongst these later, modelling tools have been developed that allow analysis of PPP transport from the site of application into the aquatic environment with further prediction of final concentrations in surface water and groundwater (PECs - Predicted Environmental Concentrations), thus estimating putative threatening scenarios to the aquatic biota and consequently for the ecosystems functioning. These modelling tools are integrated in risk assessment schemes required for the PPPs licensing process.

The modelling tool used currently in the European Union for the above purposes is the FOCUS platform[4]. FOCUS was designed and developed within several European projects following Regulation (EC) No. 1107/2009 (EC, 2009), being often used to calculate PECs in surface water and groundwater, depending on application doses and physicochemical properties of chemicals and european soils. The fate of a given substance in different environmental compartments after its application can be estimated by the application of three mathematical models: MACRO, to estimate the contribution of drainage; PRZM, to estimate the contribution of runoff; and TOXSWA, to estimate the final PECs in surface water (PECs (sw)). The tool is divided into several modules developed through a 4-level stepwise approach of sequentially increasing complexity. The first and simplest step corresponds to a simple approach using simple kinetics and assuming a loading equivalent to a maximum annual application.

The evaluation of adverse effects of PPPs on the non-target biota of the aquatic compartment, more specifically of surface waters, is important as a basis to characterize the environmental hazardous potential of these substances (e.g., Abrantes et al., 2009; Pereira et al., 2009). Currently the literature is plenty of studies evaluating the toxic potential of PPPs to aquatic non-target organisms, and some of these studies noticed an

[4] Open-access software, downloadable from: http://esdac.jrc.ec.europa.eu/projects/focus-dg-sante

increased toxicity of commercial PPPs compared to that shown by the corresponding active ingredients (a.i.s), this linking to the activity of supposedly inert formulants or adjuvants used in the formulations (Cedergreen and Streibig, 2005; Pereira et al., 2009; Vidal et al., 2016, 2011). Current registration requirements of PPPs present some flaws, including the exemption of a detailed risk assessment on formulations to better consider interactions between its components and the consequences of such interactions in the product's environmental toxicity. Still, the increased coverage of environmental safety by regulatory guidelines is undeniable. In this way, the agrochemicals industry has been forced to innovate in the formulation of its products (EC, 2009). Common strategies followed in this context have been the replacement of formulants or adjuvants by natural products or greener equivalents and/or the improvement of PPP target delivery (Cantrell et al. 2012; Dayan et al. 2009; Singh et al. 2013).

An alternative approach that has been neglected so far as to our knowledge regards the production of environmentally friendlier agrochemical formulations through the manipulation of the components ratio towards a product with lessened environmental impacts. This identified gap motivated the present study, which was based on the assessment of deviations from reference models of mixture toxicity denoting synergism, antagonism, dose-level and dose-ratio effects (see e.g. Jonker et al. 2005 for the general theory on mixture toxicity studies). We hypothesized that the combination of formulation components could be worked out to promote antagonistic effects in non-target environmental indicators. These combinations would represent eco-friendlier formulations as compared to the formulation used in the current commercial product. As a ruling principle validating the usefulness and applicability of the methodology, the eco-friendlier formulations found should maintain the efficacy against the target weeds as compared to this commercial formulation. The herbicide Winner Top® was selected as model to follow the above rationale. Inherently, the target weed *Portulaca oleracea* and the non-target organism *Lemna minor* were selected for testing.

Winner Top® is a 2-way formulation using nicosulfuron and terbuthylazine as active ingredients (a.i.s) plus undisclosed formulants (Selectis, 2012). This herbicide has been used on maize cultures to combat annual grass weeds and dicotyledonous weeds such as *Portulaca oleracea* and *Amaranthus* spp.. Its application should be performed after the emergence of the weeds, when they have up to 3-5 leaves and coincidently maize cultures have 4-6 leaves. Its action is systemic and residual by being distributed throughout the plant after being taken up through the roots and leaves. The recommended application doses are 2.5 L/ha (corresponding to 41.88 g/ha of nicosulfuron and 625 g/ha of terbuthylazine) for susceptible species such as the above (or in soils with high organic matter content) or 3L/ha (corresponding to 50.25 g/ha of nicosulfuron and 750 g/ha of terbuthylazine) for moderately susceptible species such as *Polygonum lapathifolium* and *Digitaria sanguinalis*. A pickle should be prepared for pesticide application (200-400 L/ha), and the application should be carried out once a year. Winner Top® is considered a very effective herbicide and its effects are perceptible 7-10 days after the application through a visible blocking of weeds growth (Selectis, 2016a).

Nicosulfuron and terbuthylazine belong to sulfonylureas and 1,3,5-triazines chemical groups, respectively (DGAV, 2015; Selectis, 2016a, 2012). Their modes of action

are well known and characterized. Nicosulfuron prevents the growth of susceptible plants by blocking the plant amino acid synthesis through the inhibition of acetohydroxyacid synthase (DGAV, 2015; Lewis et al., 2016) and terbuthylazine inhibits photosynthesis by acting as a photosystem II blocker (DGAV, 2015; Lewis et al., 2016).

Portulaca oleracea (common name purslane; "beldroega" in portuguese) was selected as the model target weed because it is a major target of Winner Top® (Selectis, 2016a). It is very common and easy to find in seeds since it is consumed as part of Mediterranean salads. Furthermore, it is of a relatively small size and it can be cultivated all over the year. These particular features facilitate the use of *P. oleracea* as a rapidly growing test organism in the laboratory as long as the culturing soil is kept within optimum pH ranges of 5.5-7. On the other hand, *Lemna minor* was selected as the model non-target species. This option was ruled by (i) the established status of *Lemna* sp. as standard ecotoxicological test species (e.g. OECD 2006a); (ii) the herbicidal and systemic nature of Winner Top®, which *a priori* suggests that macrophytes should be more sensitive (thus more environmentally protective) than non-plant indicators and equivalent indicators lacking a vascular system such as microalgae; (iii) the results obtained in previous studies as detailed in chapter 2, where *Lemna* sp. were indeed proven to be more sensitive than microalgae to the PPP and its a.i.s, and similar sensitivity was found between the smaller and easier to handle *L. minor* compared to *L. gibba*.

This study was structured following a tiered approach, through the accomplishment of sequential specific aims. In a first tier single chemical concentration-response curves by the non-target organism *L. minor* following exposure to nicosulfuron and terbuthylazine were obtained to feed reference mixtures toxicity models of Concentration Addition (CA; Berenbaum, 1985) and Independent Action (IA; Bliss, 1939), and further prediction of mixture toxicity response surfaces. CA assumes that mixture components act as dilutions of each other since they possess a similar toxicological mode of action. It can be represented mathematically for a mixture of *n* components by the following equation:

$$\sum_{i=1}^{n} \frac{Ci}{ECx_i} = 1$$

where C_i represents the individual concentrations of each component present in the mixture with a total effect of x% and ECx_i are those concentrations of the components that would alone cause the same effect x_i as observed for the mixture. On the other hand, IA assumes that the components of a mixture act independently (dissimilar mode of action). So, the effect of one of the components in the mixture should remain unchanged in the presence of another component. The joint effect is calculated by multiplying the probability of non-response, of each i^{th} component of the mixture. It can be mathematically expressed by the following equation:

$$Rmix = 1 - \prod_{i=1}^{n} [1 - E(Ci)]$$

where C_i represents the individual concentrations of each component in the mixture and $E(C_i)$ is the effect of Ci when the i^{th} component is dosed singly. The effective mixture concentrations are commonly presented using the dimensionless Toxic Units (TU) scaling

allowing a measure of the toxic strength. The sum of the quotients C*i*/EC_{50}*i* was applied for the purpose following the CA principles for i^{th} mixture components (see Jonker et al., 2005 for more details on the TU approach).

The predicted mixture response based on experimentally assessed responses to single a.i. exposures allowed the definition of the mixture treatments for further testing covering the whole mixture response curve, hence using non-equitoxic ratios between the components. Then, the second tier of the study comprised mixtures toxicity testing with the non-target *L. minor*, based on the predicted mixtures response surface yield in the first tier. Here we aimed specifically at defining the actual response surface of the selected non-target organism following exposure to the mixture of Winner Top®' a.i.s towards spotting deviations from the reference models of mixture toxicity. In practice, the responses obtained following exposure to the mixtures were compared to those expected by applying the reference models and the deviations were analysed through the approach by Jonker et al (2005). Deviations considered for the purposes included synergist/antagonist behaviour, dose-level and dose-ratio effects, with our major expectation being the spotting of antagonistic mixtures, which could represent eco-friendlier alternatives to Winner Top®.

The third tier of the study aimed at testing the efficacy, towards the target weed *P. oleracea*, of eco-friendlier mixtures of nicosulfuron and terbuthylazine behaving antagonistically (as assessed in tier 2) towards the non-target indicator *L. minor*.

3.2. MATERIAL AND METHODS

3.2.1. Chemicals

The chemicals used in the toxicity bioassays were the herbicide Winner Top®, marketed in Portugal by Selectis®, which is a concentrated suspension with 16.75 g/L nicosulfuron and 250 g/L terbuthylazine (Selectis®, Portugal;) and its a.i.s Terbuthylazine ($C_9H_{16}ClN_5$, CAS No.5915-41-3; Pestanal®, Sigma-Aldrich®, Steinheim) and Nicosulfuron ($C_{15}H_{18}N_6O_6S$, CAS No. 111991-09-4; Pestanal®, Sigma-Aldrich®, Steinheim). The stock solutions were prepared immediately before each assay by dissolving the a.i.s or diluting the commercial formulation in distilled water or in each test medium depending on observed solubility constraints.

3.2.2. Test organisms

3.2.2.1. *Lemna minor*

Cultures of *L. minor* were maintained in 500 mL Erlenmeyers filled with ca. 200 mL of Steinberg medium (OECD, 2006a), at 20°C with a photoperiod of $16h^{Light}$:$8h^{Dark}$ (light intensity: ≈2000 LUX), under sterile conditions. Erlenmeyers and medium were sterilized

by autoclave (60-90 min, 120°C, 1 atm). Cultures were renewed once a week under approximately sterile conditions (close to the flame).

3.2.2.2. *Portulaca oleracea*

Seeds of the terrestrial plant *P. oleracea* were purchased at a local store of plants and seeds. The seeds were separated into groups of 10 and placed in 1.5 mL Eppendorf tubes to facilitate the seeding at the beginning of the tests (Figure 1) (see section 3.2.3.3 for further details on the test protocol).

Figure 1 – Sachet of *Portulaca oleracea* seeds*;* Producer: Flora Lusitana (top panel); and the placement of their seeds in 1.5 mL Eppendorf tubes (bottom panel).

3.2.3. Bioassays

3.2.3.1. <u>Growth inhibition tests with *Lemna minor: single chemicals*</u>

Growth inhibition tests with *L. minor* followed the recommendations of the OECD guideline 221 (OECD, 2006a). Test medium (Steinberg medium) and all equipment used for the tests

(beakers, flasks, etc.) were clear of chemical contaminants by dedicated washing followed by autoclave sterilization.

Tests were carried out in disposable 6-well macroplates (final volume of each well: 10 mL) as adapted by Kaza et al. (2007) and Kolasińska et al. (2010), at 23°C, under continuous illumination (intensity: ≈1700 LUX), during 7 days. Tests started by inoculating each well with 3 healthy colonies with 3 fronds each, which were then allowed to grow as monocultures exposed to concentrations ranges of nicosulfuron and terbuthylazine individually (Table 1). The tests design included 3 replicates per test concentration and 6 control replicates. Three extra replicates were collected from cultures for determination of the average dry weight (dw) at the beginning of the test by drying until constant weight (normally 24 h at 60°C). The macroplates were moved once a day in the climatic chamber to prevent the effect of eventually non-homogeneous incubation conditions. After 7 days of exposure, all colonies were collected from each replicate and fronds were counted. Colonies were rinsed with distilled water, blotted in absorbent paper to remove excess water (the root fragments were also included) and then incubated at 60°C until constant weight (normally during 24 hours) for the determination of final dry weight. *Lemna* yield in each individual treatment was calculated for the variables 'frond number' and 'dry weight', as the difference between records taken at the end and the beginning of the test. The inhibition in yield (I_Y) was then expressed as:

$$\boldsymbol{Iy} = \frac{(\boldsymbol{Yc} - \boldsymbol{Yt})}{(\mathbf{Yc} \times \mathbf{100})}$$

where Y_C and Y_T represent the mean value of yield for the controls and the yield in each replicated treatment, respectively.

Table 1 - Concentrations of nicosulfuron (Nic.) and terbuthylazine (Terb.) used in single-chemical exposures of the macrophyte *Lemna minor* to each active ingredient composing the commercial formulation Winner Top®.

Toxic	Concentrations (µg/L)
Nic.	0; 1; 2; 3; 4; 7; 10; 17; 27; 43; 69
Terb.	0; 20; 33; 52; 84; 134; 215; 344; 550

3.2.3.2. Growth inhibition tests with *Lemna minor: mixture modelling and toxicity tests*

Frond yield experimental records following single exposure to terbuthylazine and nicosulfuron were fitted to the nonlinear decay model (Barata et al., 2006). Significant fitting was always achieved, with model accuracy being assessed through adjusted coefficient of determination (r^2) and residual distribution (Quinn & Keough, 2002); the

significance of regressions and their regression coefficients were determined by the F-test of overall significance and the t-test, respectively. Prediction of the joint action of chemicals was carried out by integrating the experimental data into the reference mixture models of CA and IA, which assume that there is no interaction between the components of the mixture while exerting toxicity. Based on the predicted response curves, a 5x5 range of concentrations for testing mixtures was rationally established (Table 2) following a composite design that covered any possible interactions between mixture components at various mixture ratios (see details in Altenburger et al. 2003).

Table 2 - Concentrations of nicosulfuron (Nic.) and terbuthylazine (Terb) used in exposures of the macrophyte to combinations of the a.i.s, in µg/L and in Toxic Units (TU). The number of replicates for each tested treatment (light grey cells) is referred within the corresponding cell (six replicates for controls and three replicates for the other concentrations).

			Chem. 2 – Terb.				
	TU		0.00	0.50	0.77	1.17	1.80
		Concentrations (µg/L)	**0.000**	**36.491**	**55.927**	**85.715**	**131.369**
Chem. 1 – Nic.	0.00	**0.000**	⑥	③	③	③	③
	0.50	**1.447**	③	③	③	③	
	0.58	**1.668**	③	③	③		
	1.17	**3.398**	③	③		③	
	1.80	**5.208**	③				③

L. minor was exposed to the established mixtures of terbuthylazine and nicosulfuron (Table 2) as described for single chemicals (see previous section 3.2.3.1). Frond yield inhibition (see section 3.2.3.1. for calculation details) was collected at the end of the test. The experimental responses were compared to the reference CA and IA models, as well as to CA and IA added the deviation functions allowing the assessment of synergic/antagonist effects, dose-level and dose-ratio dependent effects; such effects are denoted by two parameters defining the functional form of the deviation pattern, 'a' and 'b', as described in Jonker et al. (2005). Mixture toxicity modelling and analysis was run in a customized MS®Excel® spreadsheet (ToxCalcMix, version 1.0, last rev. 20/01/2016; Nogueira, unpublished data).

3.2.3.3. Vegetative vigour test with *Portulaca oleracea*

After finding antagonistic effects towards the non-target species (thus eco-friendly formulations), their equivalents in the target weed were tested, this allowing an insight on the efficacy of the selected alternatives. In this way, *P. oleracea* was tested against mixtures of terbuthylazine and nicosulfuron corresponding to the mixtures found antagonistic to *L. minor* and concomitantly resulting in less than 20 % frond yield inhibition (environmental protective benchmark; E.C.B., 2003): EC_1, EC_5 and EC_{20} estimated following the

experimental assessment of the mixtures toxicity to *L. minor* (Tables 4 and 5). EC estimation was carried out within the ToxCalcMix spreadsheet following the fitting of the response curve best describing the experimental results (IA, with deviation of *dose level* dependence type; see section 3.3.1 for details).

Since the rationale addresses the environmental effects of Winner Top®'s a.i.s in surface water by using the representative indicator *L. minor*, the transport of the pesticide residues through the terrestrial compartment where the application occurs was taken into account to calculate treatment doses applying to *P. oleracea* testing. In practice, we assumed *L. minor* mixture EC_x values as PEC (sw) equivalents and used step 1 by FOCUS to find corresponding application doses of each a.i., i.e. the actual exposure concentrations used in *P. oleracea* tests. The simplest step of the FOCUS platform (step 1) was sufficient in this case because Winner Top® should be applied only once a year (Selectis, 2012) and this particular study is not based on any specific scenario. Since FOCUS runs backwards compared to our needs (the user insert the toxic application doses values and the platform provides him the values that reach the water), a previous calibration step was necessary by simulating several application doses for each a.i., in order to calculate the corresponding PEC (sw) at day 1. The simulation parameters used for each a.i. were as presented in Table 3. Following all simulations (Table 3), a linear regression model was applied to robustly relate application doses and PEC (sw) values (Figure 2). Mixture ECx values for *L. minor*, corresponding to PECs (sw), were then converted into application doses (Table 4). Single chemical application doses were also tested in order to complete the mixtures toxicity design of the test towards the target weed and obtain more robust response curves (Tables 4 and 5).

Table 3 - Values used to complete the parametrization required at step 1 of the FOCUS platform for estimating PEC values in surface water based on pesticide application doses. All values but application doses were retrieved from EFSA (2011) for terbuthylazine and EFSA (2007) for nicosulfuron.

	Nicosulfuron	**Terbuthylazine**
Water solubility (g/L)	9.5 (pH 6.7, at 19.7 °C)	8.5 (at 20°C)
K_{OC} /K_{foc} (L/kg)	21	151
DT_{50} in soil (days)	16	19.4
DT_{50} in water/sediment system (days)	42.3	69.9
DT_{50} in water (days)	65	1000
DT_{50} in sediment (days)	14	69.9
Number of applications per season	1	1
Crop type	Maize	Maize
Region and season of application	South Europe, Mar-May	South Europe, Mar-May
Application doses (g/ha)	5, 10, 15, 20, 25, 42, 50, 75, 100	50, 100, 250, 325, 500, 625, 750

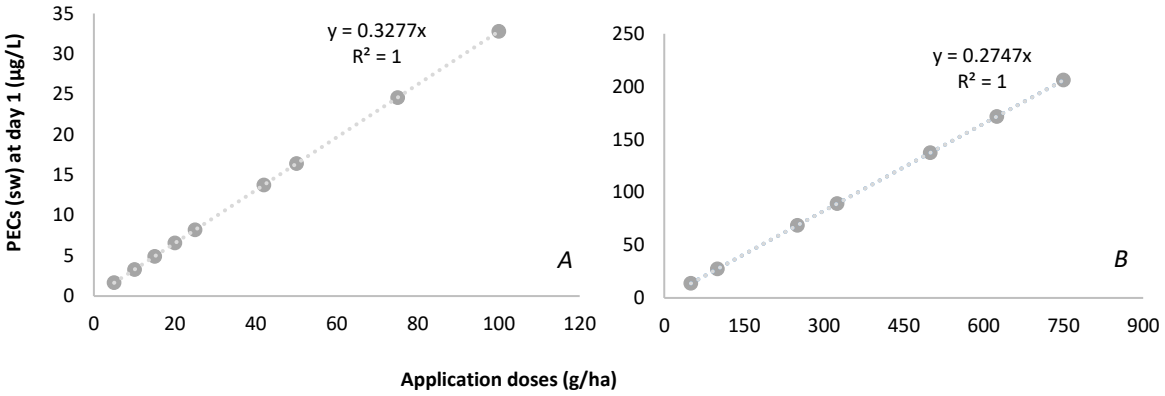

Figure 2 – Linear relationship established between application doses (independent variable) of nicosulfuron (A) and terbuthylazine (B) and corresponding PECs (sw) at day 1 obtained following simulation in FOCUS-step 1, with the respective equations and regression coefficients.

Table 4 – Transformation of PEC (sw) equivalents reflecting the effective concentrations of mixture components (nicosulfuron, Nic., and terbuthylazine, Terb.) causing 1, 5 and 20% *L. minor* yield inhibition (Mix EC_1, EC_5 and EC_{20}) into the corresponding application doses for use as exposure treatments in mixture testing with the target weed, *P. oleracea*. Single chemical application doses and corresponding PECs (sw) are also presented for consistency.

	Nic. (y = 0.3277x)		**Terb. (y = 0.2747x)**	
	PEC (y) (µg/L)	Corresponding application dose (x) (g/ha)	PEC (y) (µg/L)	Corresponding application dose (x) (g/ha)
Mix EC_1	0.292	0.891	0.951	3.462
Mix EC_5	0.720	2.197	4.793	17.448
			8.241	30
			10.988	40
			13.735	50
Mix EC_{20}	1.593	4.861	19.981	72.738
	2.458	7.5		
	4.915	15		
	9.831	30		
	19.662	60		
	39.324	120		

The full test mixture scheme used in tests with *P. oleracea* is summarized in Table 5 for clarity. Winner Top® was also tested as an additional mixtures test treatment for comparative purposes and insights on a putative effect of adjuvants on the overall pesticide efficacy. The typical application dose was down-ranged (4.861 g/ha nicosulfuron × 72.738 g/ha terbuthylazine instead of 41.88 g/ha nicosulfuron × 625 g/ha terbuthylazine; see introduction) while keeping the ratio between the a.i.s as used in Winner Top®, this allowing a direct comparison with one of the combinations between a.i.s (that marked with an asterisk in Table 5). Table 5 shows the corresponding concentrations of both a.i.s. as

well as the position of this treatment within the toxic strength (TU scaling) range applying in the mixtures toxicity test.

Table 5 - Composite design of mixture (nicosulfuron, Nic., with terbuthylazine, Terb.) testing with *P. oleracea*. Concentrations of each component of the mixture are given as application doses (g/ha) and as TU (calculated based on the two evaluated parameters: dry weight and frond number) for consistency with mixtures toxicity theory (see Introduction). The corresponding ECx values (µg/L) retrieved following the assessment of the response curves by *L. minor* to mixtures of Nic. and Terb. are also given for clarity. The number of replicates for each tested treatment (light grey cells) is referred within the corresponding cell (four replicates for controls and three replicates for the other concentrations). The asterisk marks the mixture which allowed a direct comparison with Winner Top®, hence a view on the effects of adjuvants in the overall efficacy of the pesticide.

					Component 2 – Terb.						
	ECs (*Lemna minor*) (µg/L)				CTRL 0.000	EC_1 0.951	EC_5 4.793				EC_{20} 19.981
		TU		weight	0	0.06	0.30	0.51	0.69	0.86	1.25
				number	0	0.07	0.37	0.63	0.84	1.05	1.53
		Dry	Frond	**Application doses on *P. oleracea* (g/ha)**	**0.000**	**3.462**	**17.448**	**30**	**40**	**50**	**72.738**
Component 1– Nic.	CTRL 0.000	0	0	**0.000**	④	③	③	③	③	③	③
	EC_1 0.292	0.02	0.02	**0.891**	③	③	③				
	EC_5 0.720	0.05	0.06	**2.197**	③	③	③				
	EC_{20} 1.593	0.12	0.13	**4.861**	③						③*
		0.18	0.19	**7.5**	③						
		0.36	0.39	**15**	③						
		0.71	0.77	**30**	③						
		1.42	1.54	**60**	③						
		2.84	3.09	**120**	③						

The effects of terbuthylazine, nicosulfuron, their mixture and Winner Top® on the target plant *P. oleracea* were tested following the recommendations by the OECD guideline N227 (OECD, 2006b). This guideline is used for the assessment of the effects of chemicals (general chemicals, biocides and plant protection products) on the vegetative vigour of terrestrial plants (standard plants considered in this guideline include *Zea mays, Daucus*

caeota, Lactuca sativa, etc.). Approximately 10 days prior to the beginning of the exposure, plants were grown from the seeds in plastic pots with a circular area of 95 cm^2 (Figure 3).

Figure 3 – *Portulaca oleracea* in 2-true leaf stage (approximately 5-6 days after seeding).

The pots were firstly holed at the bottom for placing a rope, which aimed the driving of water from a vessel placed under each pot into the soil for constant moistening (Figure 4). After this, the pots were filled with 200 g (dry weight) of soil. The soil used was LUFA soil, a natural soil originating from Speyer, Germany (Løkke, 1998), widely accepted as a standard matrix for soil toxicity tests. General physical and chemical features of the LUFA soil, namely pH, electrical conductivity, water content, and organic matter content were determined before experiments (Table 6) following ISO/DIS (1998), Davis & Freitas (1970), ISO (2008), SPAC (2000) and Tan (1996). These analyses confirmed the overall adequacy of LUFA for testing with *P. oleracea*, except for its too acidic pH. A trial parallel experiment was conducted to define the amount of calcium carbonate that should be added to LUFA in order to raise its pH levels to optimal pH ranges for *P. oleracea*. Based on the results of this trial, 100 mg $CaCO_3$ were added to each 200 g dw soil used to set up each replicate.

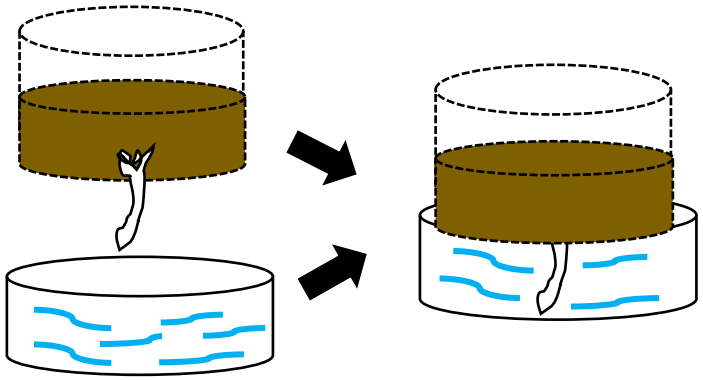

Figure 4 – Representative scheme of the experimental setting for toxicity testing with plants, in this case *P. oleracea*. A rope was attached to the bottom of each plastic pot holding the soil, for water collection from a vessel placed below the pot and permanently filled with tap water.

Table 6 – Measured physical and chemical parameters of the LUFA soil. Mean (± standard deviation) of 3 replicated samples are shown per parameter.

PH	Conductivity (µs/cm)	Dry mass (%)	Wet mass (%)	Organic matter (%)
5.323 (±0.015)	309.000 (±17.776)	6.584 (±0.088)	6.178 (±0.078)	2.322 (±0.217)

Dry mass= [(weight of moist soil – weight of dried soil)/ weight of dried soil] × 100; Wet mass= [(weight of moist soil – weight of dried soil)/ weight of moist soil] × 100; Total OM % = [(weight of dried soil – weight of soil after loss on ignition)/ weight of dried soil]×100.

The seeds were then placed near the soil surface, separated so as to form a kind of internal circle to the vessel (Figure 5), and as much equidistant from each other as possible; this prevented an uneven distribution of seeds and consequently the interference from unbalanced competition between further emerged plants within each replicate. Ten seeds were used per replicate, according to the OECD recommendations and considering the small size of the pots, and three replicates were established per treatment.

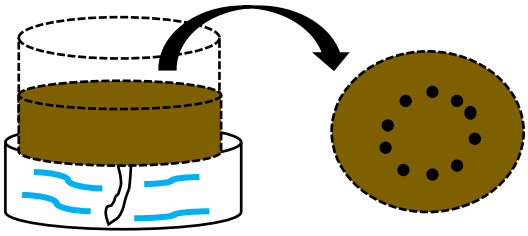

Figure 5 - Representative scheme of the seeds arrangement within each replicated pot.

Whenever necessary, the vessel was filled until ca. 150 ml to keep soil moistening constant. Nutrients (Substral®; Figure 6) were added to the water once along with the first water refiling in the vessel (14 mL/L). From seedling onwards and during the whole test period, the pots were kept at 20 ± 3°C under a 16 h^L:8 h^D photoperiod (light intensity and humidity were of 19600 lux and 50 ± 2 %, respectively). The seeds began to germinate on day 3 after seedling. Following germination, the number of plants was reduced down to 5 per replicate plot. The plants were left to grow until reaching the 4-true leaf stage (approximately 10 days), when they were pulverized with the test solutions (Table 5; Figure 7). The sprayers used for the pulverization were purchased to VWR® (Turn 'n' spray, Bürkle, 250 mL) and were standardized as to spray volume per pulverization (1.2 ±0.1 mL). On this basis, and to avoid variation in moistening between treatments that could constrain the results, the number of pulverizations used to treat all replicates was fixed (12 pulverizations per replicate, corresponding to 14.4 mL of each test solution, either of an a.i., of a mixture of the a.i.s or the control where blank tap water was used). The test solutions were worked

out considering this constraint so that the planned mixture treatments could be achieved. Each pot was pulverized individually in a Hotte and the pulverization distance was kept constant within and between replicates and treatments (Figure 7). All equipment used in the test (pots, beakers, flasks, etc.) was clear of chemical contaminants under dedicated decontamination solutions and autoclaving when appropriate.

Figure 6 – Substral®, "plant food" enriched in iron.

Figure 7 – A replicate being pulverized.

The plants were observed daily until the end of the test, at day 16 following pulverization. At the end of this period, the leaves were counted and shoots were harvested for further dry weight record after drying until constant weight at 60°C. Although the initial idea was to collect also the dry weight of roots, this parameter was unfeasible since *P. oleracea* roots are very thin and scattered (Figure 8), making any attempt to separate them from the soil unsuccessful.

Figure 8 – Roots of *P. oleracea* dispersed in the soil.

To analyse the data of the vegetative vigour test with *P. oleracea*, a one-way ANOVA followed by the post-hoc Dunnet test ($p<0.05$) was run for the components of the mixture (Nic. and Terb.) individually and to binary mixtures of them (Nic. + Terb.), in order to assess significant inhibition effects ($p<0.05$) in dry weight and leaf number relative to the controls.

3.3. Results and discussion

3.3.1. *Lemna minor*

The response of *L. minor* to single exposures allowed to define the mixture scheme for the second tier of the study (mixture toxicity testing with *L. minor*), and to draw some conclusions regarding the sensitivity of the non-target species to terbuthylazine and nicosulfuron, as detailed in chapter 2 of the present dissertation. In brief, the results showed that nicosulfuron was the most toxic a.i. to *L. minor* (see Figure 9 and Table 5 in Chapter 2), with 7 d-ECx values for yield inhibition one order of magnitude lower that the corresponding benchmark found for terbuthylazine. Significant fitting of the nonlinear decay model to the experimental results was achieved following exposure to nicosulfuron ($y= 100-[101.737/(1+(x/3.462)^{1.498})]$; $R^2= 0.949698$; t-value= 11.40) and terbuthylazine ($y= 100-[99.937/(1+(x/81.669)^{1.140})]$; $R^2= 0.965071$; t-value= 12.25). The parameter chosen for the determination of the ECx values was the frond number yield instead of the dry weight yield. This option was ruled by (i) a better fitting of the data to the allosteric decay model of frond number; (ii) the fact that dry weight could integrate interference from growth of algae observed in the frond surface of the macrophytes.

The integration of the experimental data of the mixture toxicity test with *L. minor* into the mixture models of toxicity (CA and IA, as well as CA and IA added the deviations established by Jonker et al., 2005) indicated that the model best describing the actual response surface was dose-level dependent IA, with an improved fit to the experimental data ($F = 28.287$; $p <0.001$) and lower AIC (Akaike's information criterion; Motulsky and Christopoulos 2003) value compared to the alternative models (Table 7). The predictive

ability of dose-level dependent IA in the present case is additionally illustrated by Figure 9A for clarity, where the strong association between observed responses and the corresponding responses as predicted by the model is evident.

Table 7 - Statistical parameters for the fitting of experimental data to CA and IA, as well as deviations from these reference models, denoting dose-level dependence (DL), synergism/antagonism (S/A) and dose-ratio (DR) dependence. RMSD (Root Mean-Square Deviation) provides a measure of the difference between predicted values and those actually observed (the lower the better); SSE (Error Sum of Squares) is the sum of the squared differences between each observation and its group's mean (the lower the better); SSE (df) stands for the residual degrees of freedom. AIC (Akaike's information criterion) is an alternative to statistical hypothesis testing; assessing the relative quality of statistical models in explaining a given dataset; models with lower AIC are more likely to be correct compared to others (Motulsky & Christopoulos 2003). Parameters 'a' and 'b' represent the modifications made to the baseline models CA and IA while rewriting the deviation functions to assess synergism/antagonism, dose level dependence and dose ratio dependence as developed by Jonker et al. (2005).

	CA				***IA***			
	Baseline	*DL*	*S/A*	*DR*	*Baseline*	*DL*	*S/A*	*DR*
R^2	–	0.207	0.730	0.744	–	0.747	0.669	0.695
RMSD	–	3.450	3.544	3.502	–	3.493	3.926	3.823
SSE	1300.027	892.486	954.770	907.578	1434.573	915.188	1171.477	1081.742
SSE (df)	71	69	70	68	71	69	70	68
AIC	230.794	–	–	–	238.378	-13.60	–	–
P (F-test)	–	<0.001	<0.001	<0.001	–	<0.001	<0.001	<0.001
a	–	1.100	1.750	1.194	–	0.002	1.000	0.701
b_{DL}	–	-0.338	–	–	–	-1230.555	–	–
$b_{Nic.}$	–	–	–	2.869	–	–	–	2.357
$b_{Terb.}$	–	–	–	-1.675	–	–	–	-1.656

The value found for the 'a' parameter considering the dose-level deviation of IA was slightly above zero (0.002; see Table 7), which indicates a tendency for an antagonistic behaviour of the mixture at low dose level and a synergic behaviour at high dose level (Jonker et al. 2005). This latter is unperceived in the isobologram (Figure 9B), where antagonism throughout the whole response surface at the dose range focused is rather evident by the convex shape of the isoboles. The value found for the 'b' parameter was heavily negative (-1230.5; see table 7), confirming that change from antagonism into synergism is not likely to occur, but that the magnitude of antagonism is effect level dependent (Jonker et al., 2005). Still, regardless the effect level, the isobologram (Figure 9B) denotes that stronger antagonism (higher degree of concavity relative to the origin) is found consistently at lower nicosulfuron doses. At combined doses below 1 TU of nicosulfuron and 1 TU of terbuthylazine, nicosulfuron appears to be the major responsible for the occurrence of antagonism. At combined doses of 1-2 TU nicosulfuron and 1-2.7 TU of terbuthylazine, terbuthylazine is the major responsible for the occurrence of this deviation. Overall, from ≈0.15 TU of nicosulfuron and ≈1 TU of terbuthylazine, combinations with higher concentrations of terbuthylazine and lower concentrations of nicosulfuron appear to be more antagonistic (Figure 9B).

The antagonistic behaviour of the mixture throughout the entire response curve was further confirmed by the significant fitting (F = 23.603; p <0.001) of the model

comprising antagonistic/synergistic deviation for IA; the positive 'a' value as shown in Table 7 reveals that antagonism occurs rather than synergism (Jonker et al., 2005). It is not uncommon that different models of interactive mixture toxicity fit significantly the experimental data (see e.g. Silva et al. 2016), especially when such a clear trend as that found in the present study is shown by the experimental data.

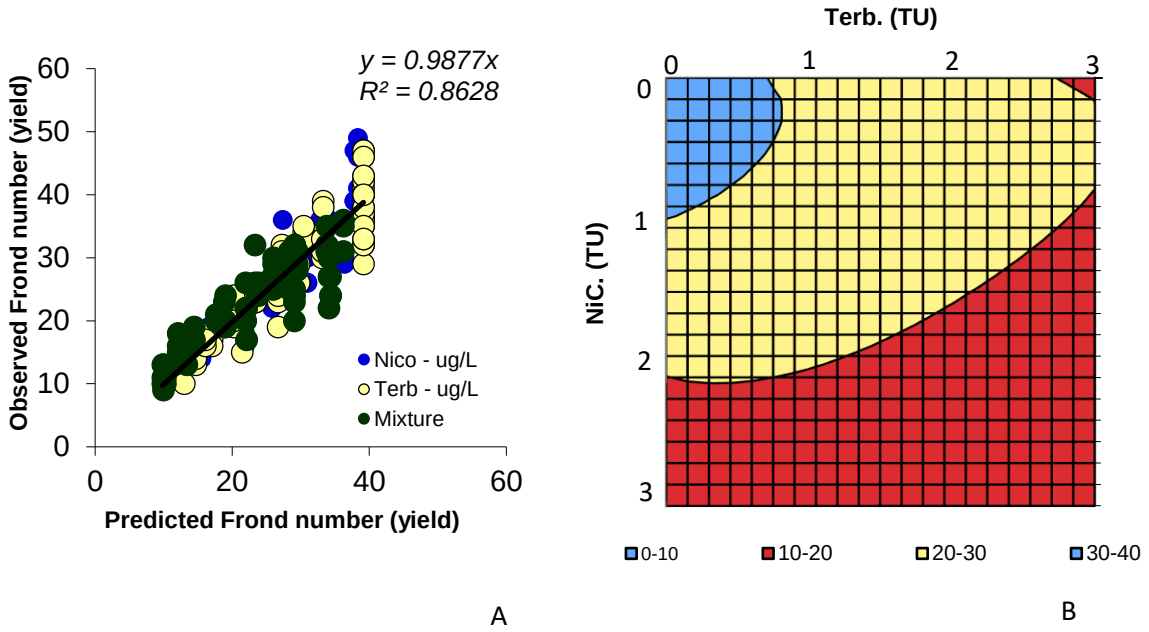

Figure 9 – Modelling of the effects in *L. minor* frond number following a 7-d exposure to combinations between terbuthylazine and nicosulfuron, according to a dose-level dependent IA behaviour. Panel A shows the relationship between observed responses (Y) to terbuthylazine (Terb.), nicosulfuron (Nic.) or their mixture and the responses predicted by the dose-level dependent IA model of mixtures toxicity (X). The points in panel A relate the variables and the straight black line represents the linear regression between the two variables facilitating the interpretation of the association between observed and predicted values; its mathematical equation and the regression coefficient (r^2) are given embedded in the figure. Panel B shows the isobologram reflecting predicted *L. minor* frond number responses to the a.i.s mixture (TU scaling shows the mixture strength) following the dose-level dependent IA model, which was that better fitting the experimental mixture toxicity dataset.

Although expert judgement depending on the goals and rationale of each study is critical to a final decision on the model that best describes the dataset, comparative fitting success analysis provides the statistical support for the purpose. The larger amount of unexplained variance (higher error sum of squares, SSE, and higher root mean-square deviation, RMSD) provided a raw indication on the better adequacy of dose level-dependent IA compared to IA antagonism to explain the mixtures toxicity (Table 7). Then, the determined AIC (see Motulsky & Christopoulos (2003) for more details) showed that the dose-level dependent IA model was more likely to reflect the mixture toxicity than the deviation of antagonism to the IA model.

Consistently with their known dissimilar mechanisms of toxic action, nicosulfuron and terbuthylazine act independently in the non-target organism *Lemna minor* when dosed as a mixture (general Independent Action behaviour). Nicosulfuron is known to affect aminoacid synthesis by inhibiting acetohydroxyacid synthase, and terbuthylazine acts as a

photosystem II blocker (DGAV, 2015; Lewis et al., 2016). Because both metabolic pathways are common to target weeds and the non-target organism tested, the noticed dissimilar mode toxic action was expected. Still, simple response addition (reference IA behaviour assuming no interactive effects due to the mixture of the components) did not describe properly the experimental responses by *L. minor*, but rather one component seems to repress the toxic potential of the other. Interestingly, given that the non-target model and target weed share the metabolic pathways involved (see above), similar antagonistic effects of the combination of a.i.s within the commercial formulation in the latter are reasonable to hypothesize, unless the susceptibility of the target weed to the mixture is modulated by a non-identified mechanism. In fact, no information was found in literature on the mechanisms that are involved in maize (major crop protected by Winner Top®) tolerance to Winner Top®, which could help to clarify the option for formulating two products that act antagonistically in susceptible plants. However, some information on the tolerance of the crop to one of its two a.i.s (nicosulfuron) was found. Some maize hybrids present tolerance to nicosulfuron (Cavalieri et al., 2008), which is related to different rates of metabolization and absorption/translocation of this a.i. in the plants (Carey et al., 1997). Tolerant hybrids rapidly detoxify nicosulfuron, transforming it into non-phytotoxic compounds by the action of cytochrome P450 monoxygenase, through hydroxylation and glycosylation reactions (Fonne-Pfister et al., 1990). A similar (but unconfirmed as to our knowledge) enhanced metabolism of nicosulfuron in *L. minor* could contribute to explain the antagonist behaviour of the mixture between the two a.i.s towards the non-target species.

Although no previous studies were found regarding the toxicity of mixtures between sulfonylureas (such as nicosulfuron) and triazines (such as terbuthylazine) for a more direct discussion, antagonistic effects of pesticide mixtures are not rare. For example, Brodeur et al. (2016) assess the interactions occurred in equitoxic and non-equitoxic binary mixtures of two formulations of glyphosate and cypermethrin to the fish *Cnesterodon decemmaculatus*. They observed that these mixtures were clearly antagonistic for all combinations tested and concluded that the antagonism was the result of a strong inhibition of cypermethrin toxicity by glyphosate. Kunce et al. (2015) evaluated the combined effects of pyrethroids and neonicotinoids on the larval development and survival of *Chironomus riparius* following a 1-h pulse exposure and they found indirect evidences for antagonism by noticing that none of the deleterious effects appeared to be amplified by the pesticide combinations. Zhang et al. (2014) tested a quaternary mixture system with different ionic liquids, which are not typically used as pesticides but bear some molecular similarity with several new generation pesticides (long carbon-based alkyl chains). They proved that one of the components ($CH_3(CH_2)7OSO_3$) of the quaternary mixture systems was consistently responsible for reducing the interaction between mixture components, hence for the induction of an antagonistic behaviour.

Our results hence fit the apparent trend highlighted in the literature for an antagonistic behaviour of mixtures between pesticides, despite synergistic interaction would be the most reasonable expectation given that Winner Top® was supposedly developed for an improved efficacy compared to single a.i. formulations and macrophytes share the targeted metabolic pathways with the weeds. The physiological or toxicological reasons for this to happen are unknown and further discussion can be made only at a

theoretical level, hence being largely speculative. Nicosulfuron is likely to be more readily uptaken by *L. minor* (systemic and contact absorption) than terbuthylazine as discussed in chapter 2, thus it may more rapidly reach the target metabolic pathway and start earlier impairing amino-acid synthesis. Still, because the exposure lasts for 7 days, there is room for a response by the cells through the synthesis *de novo* of acetohydroxyacid synthase, triggering a compensatory mechanism for better coping with the toxicant insult. Terbuthylazine should theoretically take longer to block photosystem II, slowly preventing the course of photosynthesis. Thus, the availability of the necessary energy to feed *de novo* protein synthesis is reasonable to hypothesize, supporting the above argument and possibly the refurnishing of photosystem II complexes (pseudo-symmetric heterodimer of two homologous proteins D1 and D2; Rutherford & Faller, 2003) so far unbound to terbuthylazine and hence functional.

3.3.2. *Portulaca oleracea*

The results of the exposure of *P. oleracea* to the nicosulfuron and terbuthylazine individually showed that the species was more sensitive to nicosulfuron with a lower 16 d-EC_{50} value and non-overlapping confidence intervals for the parameter dry weight (Table 8). Dry weight was the parameter that allowed to calculate more reliable EC_{50} values, since the fitting of the experimental data to the allosteric decay model was better than when using leaf number, with consequently shorter 95% confidence intervals.

Table 8 – Estimated effective concentrations (g/ha) of the a.i.s inducing 50% inhibition in aerial dry weight and leaf number (ECs_{50}) of *Portulaca oleracea* after 16 days exposure to terbuthylazine (Terb.) and nicosulfuron (Nic.),with the respective 95% confidence intervals within brackets. .

	EC_{50} (g/ha)	
	Nic.	Terb.
Dry weight	**42.221** (34.348-50.094)	**58.220** (52.443-63.997)
Leaf number	**38.844** (19.353-58.335)	**47.410** (29.344-65.476)

Consistently, single exposure to nicosulfuron and terbuthylazine significantly depressed *P. oleracea* growth either based on dry weight or leaf number records (Table 9; Figure 10 A,B). The mixture between the a.i.s was also able to significantly depress the species growth regardless the parameter used in the analysis (Table 9; Figure 10 A,B).

Table 9 – One-way ANOVA summary (df, degrees of freedom) regarding the dry weight and the leaf number of *P. oleracea* following a 16-d exposure to nicosulfuron (Nic.) and terbuthylazine (Terb.) individually and combined in binary mixtures of them (Nic. + Terb.).

Source of variation	df	$MS_{residual}$	*F*	*P*
Dry weight				
Nic.	10, 17	0.7235	15.01	<0.001
Terb.	6, 15	0.6953	28.74	<0.001
Nic. + Terb.	10, 8	0.2437	13.65	<0.05
Leaf number				
Nic.	10, 17	10547	11.48	<0.001
Terb.	6, 15	9669	8.18	<0.001
Nic. + Terb.	10, 8	3177	10.80	<0.05

The graphs of Figure 10 A,B were drawn using the dimensionless TU scaling for the treatments strength (calculation principles were described in the introduction section) to allow a direct comparison between the efficacy of single and mixture treatment. An important outcome immediately allowed by this graphical representation is that significantly impairment of *P. oleracea* growth is achieved at lower terbuthylazine toxic strength (lower TU) for dry weight (Figure 10 A) or similar single chemical and mixture treatment toxic strengths for leaf number (Figure 10 B). This puts into question the actual advantage of dosing a mixture as used in Winner Top® rather than one of the a.i. singly for the control of the weed. Terbuthylazine appears to cause a growth stimulating effect close to 0.5 TU. Around 0.7 TU nicosulfuron appears to be a better inhibitor than terbuthylazine, however a shift occurs from 1 TU onwards and terbuthylazine becomes the major inhibitor, with an abrupt decline in average dry weight being observed between 0.9 TU (dry weight average: 1.09 g) and 1.25 TU (dry weight average: 0.18 g). Around 1.4 TU, nicosulfuron was more efficient than the mixture combining 72 g/ha of terbuthylazine and 4.86 g/ha of nicosulfuron (Figure 10 A). A similar pattern was noticed when assessing leaf number (Figure 10 B). From around 1 TU onwards, terbuthylazine was the best inhibitor compared to the mixture and single nicosulfuron. At 1.6 TU the inhibition of leaf number caused by the application dose of 72 g/ha of terbuthylazine was much greater than that caused by 60 g/ha of nicosulfuron. This inhibition caused by 60 g/ha nicosulfuron was very similar to that caused by the mixture containing 72.74 g/ha of terbuthylazine and 4.86 g/ha of nicosulfuron at 1.7 TU. In general, the results indeed suggest that that there was no relevant gain in weed growth inhibition, thus treatment efficacy, by combining the two active ingredients. Actually, single terbuthylazine or, to a lessened extent, nicosulfuron

seem to be more efficient than the mixture in controlling the weed, particularly from treatment doses of 1 TU onwards.

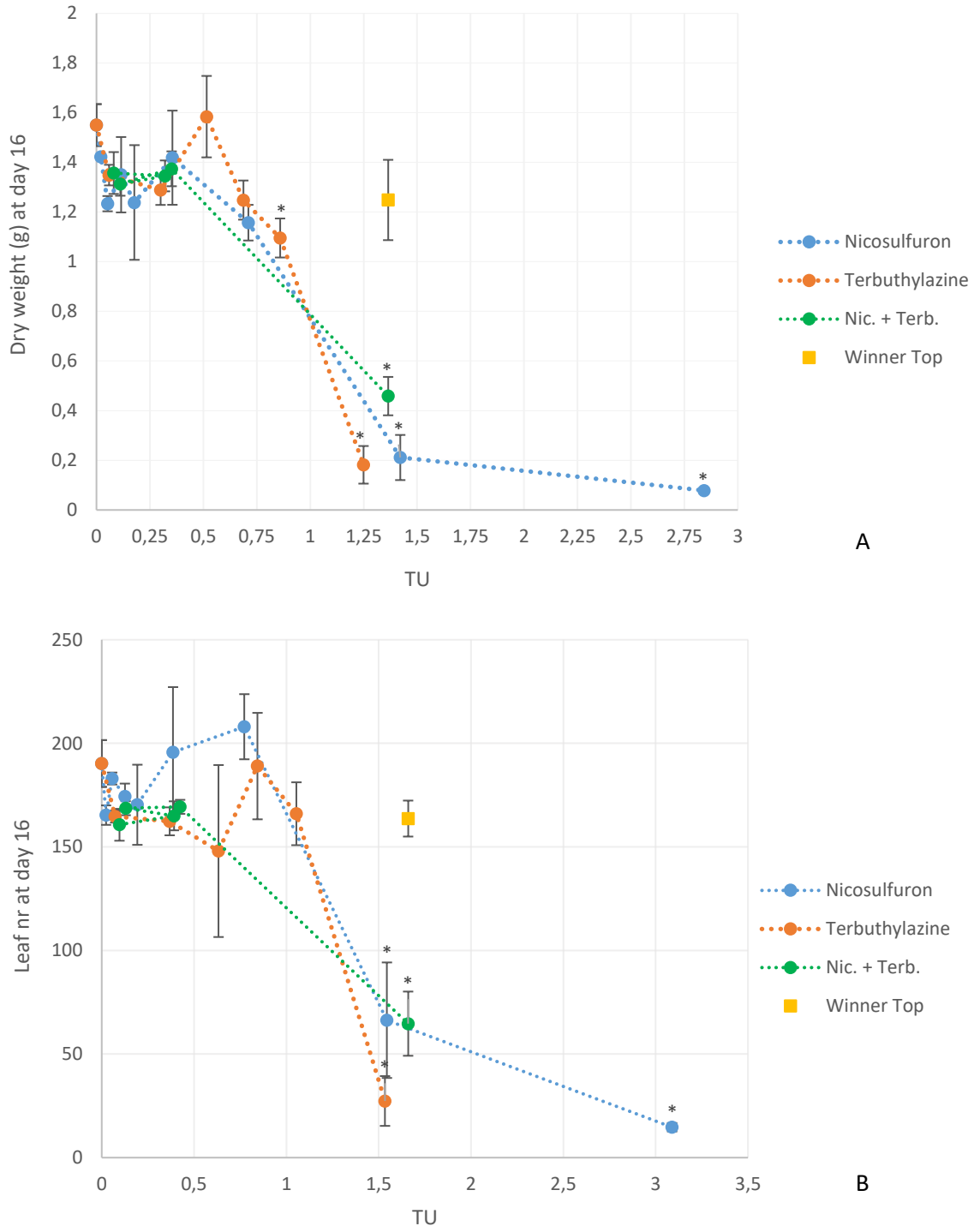

Figure 10 – Dry weight average (A) and leaf number average (B) of *P. oleracea* exposed to the components of the mixture (nicosulfuron and terbuthylazine) individually and to binary mixtures of them (Nic. + Terb.) (n=3). Error bars stands for the standard error. The dashed lines connect the mean of experimental values and they do not represent the fit to any predictive model. The asterisks assign significant differences in dry weight and leaf number relative to the control (Dunnet test; $p < 0.05$).

Also remarkable is the much lower inhibition caused by Winner Top® compared to the equivalent mixture combination of its a.i.s. (both combining 72.74 g/ha of terbuthylazine and 4.86 g/ha of nicosulfuron), either regarding dry weight or leaf number (Figure 10 A,B). In fact, Winner Top® did not significantly impaired *P. oleracea* growth while the equivalent a.i. mixture did. This response pattern was common to that retrieved in chapter 2 while testing the role of formulants in Winner Top®' toxicity towards non-target aquatic species (see *section 2.3.* in chapter 2). In summary, the formulants appear to greatly decrease the efficacy of the pesticide towards the target weed, further suggesting that the option for a mixture formulation as used in Winner Top® was not rationally equated.

As applied singly, nicosulfuron induced significant decrease in leaf number only at 1.5 and/or 3 TU and in dry weight only at 1.42 and/or 2.84 TU, corresponding to application doses of 60 and 120 g/ha, respectively (Figures 10 A,B; Table 5), which translate into surface water concentrations higher than the *Lemna minor* EC_{20} (safety) benchmark. Terbuthylazine significantly impaired *P. oleracea* leaf number and dry weight at application doses corresponding to values lower than EC_{20} to *Lemna minor*, i.e. at 1.5 TU for leaf number and 0.85 and/or 1.2 TU for dry weight, meaning 50 and 72.74 g/ha, respectively (Figures 10 A,B; Table 5). The only mixture of a.i.s able to significantly impair leaf number and dry weight was that containing the maximum dose of terbuthylazine and corresponding to EC_{20} values of the two a.i.s. estimated following single chemical testing with *Lemna minor* (Figures 10 A,B; Table 5). This suggests that terbuthylazine alone would exert better herbicidal toxicity than a two-way formulation added nicosulfuron. In fact, an eco-friendlier alternative to Winner Top® would be a formulation with terbuthylazine as the single a.i. at much lower concentrations than those used in Winner Top®. At application doses of 72 g/ha terbuthylazine (delivering the *L. minor* EC_{20} in surface waters), *P. oleracea* dry weight was reduced down to practically 0 (0.2 g) and the leaf number was reduced by 86% compared to the control treatment. In addition to exaggerated, environmentally unsafe concentrations of terbuthylazine, Winner Top® comprises nicosulfuron, at useless concentrations.

The recommended application doses of Winner Top® over *P. oleracea* weeds as mentioned in its label (see introduction) involve a nicosulfuron dose of 41.88 g/ha. This dose is comprised between two treatments tested in this study, 30 g/ha and 60 g/ha. Nicosulfuron alone did not exert significant toxicity at 30 g/ha while the opposite happened for the application dose of 60 g/ha, but both correspond to contaminant concentrations in surface water, which are way above the *L. minor* EC_{20} safety benchmark. Based on our results, a formulation composed only by nicosulfuron with a recommended similar application dose (close to 72 g/ha) would also be effective against the target species but it would again disregard environmental safety. Then, this would not be an eco-friendly alternative. Curiously, there is already in the market a Selectis® formulation containing only nicosulfuron as active ingredient - Winner® (Selectis, 2016b). This formulation has a concentration of 40 g/L of nicosulfuron and its recommended application dose mentioned on its label is 1-1.5 L/ha which corresponds to 40-60 g/ha of nicosulfuron. In our study no significant effects relative to the controls were observed for the application dose 30 g/ha

of nicosulfuron in *P. oleracea*, a very susceptible species, but only for the next higher application dose tested - 60 g/ha.

The combination of terbuthylazine and nicosulfuron is actually favourable to the non-target biota considering the results obtained with *L. minor*, but the active ingredients would have to be mixed in lower concentrations than those used in Winner Top® so that environmental safety could be ensured, i.e. not above doses implying PECs (sw) values above the *L. minor* EC_{20} (environmental protective benchmark; E.C.B., 2003). However, the mixtures established under this safety principle were not effective to combat the weed. Commercial PPP formulations are supposed to be designed towards the best performance against target pests or weeds, and in fact the two a.i.s are characterized as synergists on the Winner Top® label (Selectis, 2016a). However, this assumption was proven wrong in this study, since synergism did not occurred but the tested mixtures seem rather to have behaved antagonistically towards *P. oleracea* whenever able to significantly impair the growth of the weed (72.74 g/ha of terbuthylazine and 4.86 g/ha of nicosulfuron).

This seems to indicate that formulations are not properly tested before they enter to the market. Alternatively, one may speculate that the use of high concentrations of terbuthylazine in the formulation was related to its very poor solubility in water (6.6 mg/L; Lewis et al., 2016), which would have forced the agrochemical industry to use organic solvents to dissolve the active ingredient. To compensate for the addition of these solvents, which seem to decrease the herbicide toxicity (see Figures 10 A,B and related discussion), higher concentrations of terbuthylazine were required to achieve the necessary efficacy of the pesticide. This could be avoided with the use of other solvents or techniques, as well as by applying a structured testing protocol at the laboratory scale with non-target indicators and the pest that could provide feasible indications for the product development pipelines.

3.4. Conclusions

The outcome of this study was somewhat surprising in many ways. First of all, the combination of terbuthylazine with nicosulfuron as used in the focused commercial PPP Winner Top® behaved antagonistically towards the sensitive non-target indicator *L. minor*. In general, this would mean that the commercial product was eco-friendly since the combination of the two a.i.s would be less environmentally toxic than the equivalent single dosing of one of the a.i.s. However, the concentrations of the combined a.i.s in the commercial formulation at the recommended application doses clearly deliver PECs (sw) which are above the environmental protection benchmark determined based on single a.i.s testing with *L. minor*. The development of eco-friendlier alternatives is thus an actual need. Inherently surprising was the finding that the combination of terbuthylazine and nicosulfuron also behaved antagonistically towards the target weed, with nicosulfuron apparently being useless at treatment dosage. This is converse to the information provided in the Winner Top® label and to the generally assumed objectives of improving the product efficacy while working out its formulation. Terbuthylazine alone was more effective against

the weed than equivalent combinations with nicosulfuron (i.e. combinations using similar terbuthylazine concentration). Nicosulfuron alone was only effective against the pest when dosed at environmentally hazardous concentrations. Thus, our structured assessment demonstrated that a PPP formulation using only terbuthylazine would be an eco-friendlier alternative to Winner Top® with even enhanced efficacy against its major target weed *P. oleracea*. This alternative formulation with about 10-fold lower concentration of terbuthylazine compared to Winner Top® would result in a maximum 20 % deleterious effects on non-target aquatic organisms. Concomitantly, it would certainly have lower production costs by saving the investment on the addition of a second a.i. and avoiding unnecessary investment in higher terbuthylazine loads. Since formulants were shown to decrease the environmental toxicity and the efficacy of the commercial formulations, they constitute an additional variable that is worth considering in structured biological testing during product development.

The manipulation of the components ratio within the commercial formulation can be an innovative solution to rule the formulation of commercial products. Our dataset demonstrates that efficacy gains can be achieved with concomitant reduction of environmental hazardous potential. Furthermore, eco-friendlier, less costly alternatives to current commercial formulations can be developed. The will of the agrochemicals industry is certainly necessary for a wider acceptance of this solution as a formulation development protocol. It is true that some investment in specialized assessment at a laboratory scale would be required while mathematical modelling of putative chemical interactions between formulants should likely be the most common protocol for formulation development. However, the modelling tools we used, FOCUS and mixture modelling tools are of free web access, with FOCUS in particular being currently used by the industry for the preparation of marketing authorization dossiers. Standard toxicity testing with sensitive environmental indicators is also available in several professional and academic laboratories that outsource their services, including some currently involved in regulatory assessment of PPPs. Furthermore, single chemical toxicity testing is frequently unnecessary since a wide ecotoxicity database for different non-target environmental indicator species and for different toxicants is available, either in the scientific literature or compiled by or with the support of regulatory agencies (e.g. USEPA ECOTOX database[5]; EU Pesticides database[6]; Pesticide Properties Database[7]).

[5] https://cfpub.epa.gov/ecotox/ecotox_home.cfm
[6] http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/public/?event=homepage&language=EN
[7] http://sitem.herts.ac.uk/aeru/ppdb/en/

3.5. References

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CHAPTER 4 – Final remarks

The present work was developed under the context of the environmental hazardous potential of Plant Protection Products (PPPs) and addressed several specific objectives (please see *section 1.4* in chapter 1*)* that were fully achieved. Relevant ecotoxicological information on short-term effects of the herbicide Winner Top® and its a.i.s nicosulfuron and terbuthylazine towards selected standard non-target organisms was delivered. EC_{10}, EC_{20}, EC_{50} values and respective 95% confidence intervals, as well as LOEC values were determined regarding each test outcome and can add to the ecotoxicological database on the environmental effects of these contaminants in the aquatic compartment. Full concentration-response curves representing the relationship between the exposure to each toxic and the respective inhibition in biomass yield (%) were also obtained for microalgae and macrophyte species. The results allowed us to rank the sensitivity of the tested non-target organisms and to complete the available body of knowledge about the ecotoxicity of the focused compounds, which was actually found quite incomplete. Importantly, we found that known environmental concentrations of terbuthylazine and nicosulfuron are slightly below the concentrations that induced significant deleterious effects on the tested species, but the surface water burden has apparently been following an increasing trend. This highlights the actual environmental hazardous potential of the pesticides and adds relevance to our results. Still, studies evaluating the occurrence of these compounds in surface waters are very scarce and further, wider monitoring is required for a full picture on whether terbuthylazine and nicosulfuron represent a real hazard to the environment.

Overall, the dataset allowed concluding that Winner Top® and its a.i.s nicosulfuron and terbuthylazine significantly affected the growth of the tested species, despite the toxic response or sensitivity between macrophytes and microalgae was quite distinct. Terbuthylazine was the strongest growth's inhibitor for the two algal species and nicosulfuron was the strongest growth's inhibitor for the two macrophyte species. Contrarily to our expectations due to the systemic nature of Winner Top®'s a.i.s, the macrophytes were not fully distinguished as the most sensitive organisms to the herbicide - the microalgae *R. subcapitata* presented a similar sensitivity compared to the macrophyte *L. gibba*. Although somewhat unexpected, the finding was supported by the literature studies, and the interplay between the pesticides physico-chemical properties and the uptake routes involved is likely to explain it. There were also great differences concerning the response of different species within the same group of organisms (two macrophytes of the same genus and two different microalgae species) to each pesticide. *R. subcapitata* was much more sensitive to terbuthylazine than the other microalgae species tested, *C. vulgaris,* and *L. gibba* was the most sensitive macrophyte species to the same active ingredient. Actually, the sensitivity of *R. subcapitata* was closer to that of *L. gibba* than to that of the other microalgae species. Regarding the exposure to nicosulfuron, *C. vulgaris* was much more sensitive than *R. subcapitata* and *L. minor* was more sensitive than *L. gibba.* Regarding Winner Top®, *R. subcapitata* was much more sensitive than *C. vulgaris* and *L. gibba* was much more sensitive than *L. minor.* Finally, considering the mixture of a.i.s respecting the ratio used in Winner Top®, *R. subcapitata* was also much more sensitive than *C. vulgaris* but *L. minor* was much more sensitive than *L. gibba.* Overall, these

evidences indicate that the selection of only one species per group for studies intending to evaluate the toxicity of a particular substance may not comply with the precautionary principle since a very similar alternative may hold significantly higher sensitivity.

Also evident was that the formulants other than the a.i.s used in the commercial formulation Winner Top® bear a protective effect towards the tested non-target species. This was evidenced by comparing the impacts of Winner Top® with those by an equivalent customized mixture of the a.i.s respecting the ratio used the commercial formulation. In particular, inert ingredients were shown to rather be non-inert, and they actually seem to mitigate the toxicity of the active ingredients. Innovatively, and unlike most published studies, here the commercial formulation was compared with itself but free of formulants, thus allowing a more direct view of the true contribution of the formulants to the toxicity commercial product. Taking into account these and other evidences recorded in literature where the formulants or adjuvants were proven to have a role in modulating the PPP toxicity, and considering that formulants other than active ingredients with pesticidal activity often represent a significant part of the commercial product, it is worth reinforcing the importance of studying the environmental effects of PPP formulations as a whole. Actually, the effects of formulants are an up-date topic in the specialized discussion within the scientific community. Discussion outcomes suggest that competent authorities and regulatory agencies need to act towards a more stringent PPP legislation that properly covers un-known ingredients and their possible interactions with other formulation components within PPPs environmental risk assessment frameworks.

Our observations and conclusions can integrate the body of evidences pushing the agrochemicals industry towards the production of environmentally friendlier formulations. This links to the fourth goal of the present dissertation, which was also fully achieved. The ecotoxicity of the binary mixture between the active ingredients of Winner Top®, terbuthylazine and nicosulfuron, was assessed using the relevant non-target indicator *L. minor*. The mixture behaviour was according to dose-level Independent Action, with occurrence of antagonism being noticed throughout the whole mixture response surface. Antagonistic combinations between the active ingredients causing up to 20% impairment in *L. minor* growth (i.e. tolerable exposure levels) were tested against a target weed, *Portulaca oleracea*. These combinations, representing eco-friendly alternative ratios between active ingredients, were compared to the ratio used in the commercial formulation Winner Top®. Such a rational manipulation of the ratio between formulation components, based on expected environmental effects, was found successful as an alternative for the production of environmentally friendly formulations. The efficacy of the formulation affecting the target weed the most was higher than the efficacy of the commercial formulation. This environmentally friendly alternative formulation was composed only by terbuthylazine at about 10-fold lower concentration compared to that used in a tested Winner Top® treatment. It became evident that nicosulfuron did not add any relevant potency (against the target weed) to the formulation at the levels tested. Overall, these results are absolutely novel and suggest that the rational manipulation of the ratio between formulation components can be an effective and low-cost opportunity for agrochemical industries that aim to develop environmentally friendlier formulations as a reply to increasingly restricted legislation.

Many avenues were opened by this work, which are worth pursuing in the near future. More tests intending to evaluate the effects of nicosulfuron, terbuthilazine and Winner Top® towards other representative species could be done in order to complete the available ecotoxicity database for these toxicants; by generating the necessary data allowing the development of integrative assessment tools such as Species Sensitivity Distributions (SSDs), the environmental hazardous potential of the substances will become much better characterized and protective benchmarks much more feasibly established. The update on environmental levels of nicosulfuron and terbuthylazine is another important route allowing a better calibration of the actual environmental hazard scenario regarding these contaminants. Moreover, the same type of tests as done with Winner top®, intending to compare the commercial formulation with itself but free of formulants, could be performed with other commercial formulations, in order to gain an insight on the contribution of the formulants for the toxicity of other commercial products. The last tier of this study could also be repeated with other sensitive non-target organisms and with other target organisms of Winner Top® to test the true potential of this new methodology in ruling the formulation of environmentally friendlier alternatives to this commercial product. Validation of the methodology focusing other commercial formulations, at least considering herbicides and fungicides as important sale hits worldwide, is definitively worth considering.