Effects of plant protection products application on the quality of vineyard soils from Douro Region

Beatriz Neto Fernandes

Agricultural Engineering

Department of Geosciences, Environment and Spatial Plannings

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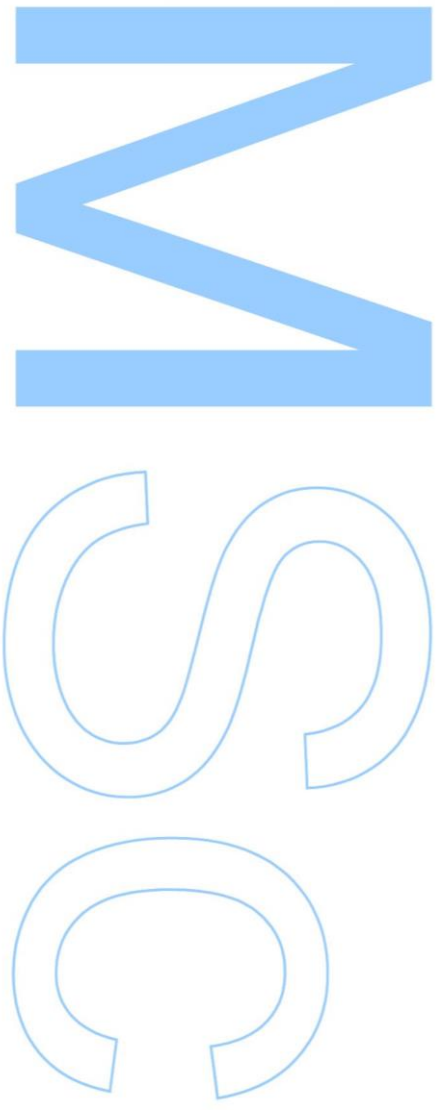
Supervisor

Anabela Ferreira de Oliveira Cachada, Researcher, CIIMAR-UP

Co-supervisor

Ruth Maria de Oliveira Pereira, Assistant Professor, GreenUPorto & Department of Biology, Faculty of Sciences, University of Porto



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pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

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Abstract

The viticulture from the Douro Region is a very important activity since it supports and links two different sectors: agri-food and tourism. In order to improve productivity, protect from losses and combat disease vectors, plant protection products (PPPs) are inevitably applied. However, these products can lead to adverse effects in the supporting terrestrial ecosystem and to adjacent aquatic systems through leaching or surface runoffs resulting on the contamination of water and soil, thus affecting aquatic and terrestrial organisms and trophic chains. One of most applied products in vineyards soils is glyphosate (GLY), an herbicide with worldwide representation. However, concerns about its environmental safety were recently raised, with a lot of divergence between studies on its non-target toxicity, namely in cover crop species used in vineyards, which have a crucial role on maintaining agroecosystems functions and in preventing soil erosion. However, it is not the only PPP product applied in vineyards, whose phytosanitary treatments include many other compounds that also account for the negative impacts. Therefore, this work has three main objectives: i) evaluate the growth and physiological responses of a cover plant species (*Medicago sativa L.*) exposed to increasing concentrations of a GLY-based herbicide (GBH), particularly focusing on the oxidative metabolism to better understand how this herbicide may compromise ecological measures recommended to protect agricultural soils due to effects on non-target plants, ii) evaluate if the application of pesticides, in general, in the Douro Region compromises soil quality, more precisely soil functions (provision of habitat, retention of contaminants and biomass production) and, iii) to have insights on the possible recovery of soils at the end of each annual cycle of phytopharmaceutical treatments. For the first objective, a laboratory experiment was conducted in which an artificial soil was spiked with increasing concentrations of GLY, and to which seeds of *M. sativa* were added. The growth of roots and shoots of plantlets was affected, being this effect accompanied by a rise of lipid peroxidation, suggesting the occurrence of oxidative stress, and by an activation of the antioxidant (AOX) system, showing that GBH-contaminated soils may pose a risk to the survival of non-target plants in the most contaminated soils. For the second and third objectives, samples were collected in two vineyards located in the Douro Demarcated Region under integrated production mode (Quinta dos Aciprestes and Quinta do Casal da Granja) throughout the year of 2018 (February, April, and June) and in January 2019. For the soil quality evaluation, physical-chemical (e.g.: pH; electric conductivity; organic matter, pseudo-total concentration of major and trace elements and synthetic organic PPPs), and biochemical indicators (enzymatic activity of dehydrogenase, arylsulfatase, phosphatase, and N mineralization) were measured. Finally, to understand if PPPs

application affected soil functions, ecotoxicological assays were performed with terrestrial (*Eisenia fetida* and *Medicago sativa*), and aquatic (*Aliivibrio fischeri*, *Raphidocelis subcapitata and Lemna minor*) organisms. The results showed that the effects observed for some parameters cannot be attributed solely to the application of PPPs since the physical and chemical properties of the soil also seem to contribute to these effects. However, even though PPPs seemed to affect soil quality at the time of application, the system appears to be able to recover from this application over time.

Keywords: alfalfa; antioxidant system; Douro demarcated region; ecotoxicological assays; fungicides; glyphosate; herbicides; integrated production mode; oxidative stress; reactive oxygen species

Resumo

A viticultura da Região do Douro é uma atividade muito importante uma vez que suporta e relacionada dois sectores diferentes: agroalimentar e turismo. De forma a aumentar a produtividade, evitar perdas e combater vetores de doenças, são inevitavelmente aplicados produtos fitofarmacêuticos (PF). No entanto, estes produtos podem causar efeitos negativos no ecossistema terrestre assim como nos sistemas aquáticos através de lixiviação ou escoamentos superficiais resultando na contaminação da água e do solo afetando assim organismos aquáticos e terrestres e cadeias tróficas. Um dos produtos mais aplicado em solos das vinhas é o glifosato (GLI), um herbicida com uma representação mundial. No entanto, têm surgido preocupações sobre a sua segurança ambiental com muita divergência entre os estudos sobre a sua toxicidade não alvo, nomeadamente em espécies de plantas de cobertura utilizadas na vinha, que têm um papel crucial na manutenção das funções dos agroecossistemas e na prevenção da erosão do solo. No entanto, este não é o único PF aplicado nos solos das vinhas, cujos tratamentos fitossanitários incluem muitos outros compostos que também são responsáveis por efeitos negativos. Assim, este trabalho tem três objetivos principais: i) avaliar o crescimento e as respostas fisiológicas de uma espécie de planta de cobertura (*Medicago sativa* L.) exposta a concentrações crescentes de um herbicida à base de GLI (GBH), principalmente com foco no metabolismo oxidativo de forma a se perceber como este herbicida pode comprometer as medidas ecológicas recomendadas para proteger os solos agrícolas devido aos efeitos em plantas não-alvo, ii) avaliar se a aplicação de PF, em geral, na Região Demarcada do Douro compromete a qualidade do solo, mais precisamente as funções do solo (provisão de habitat, retenção de contaminantes e produção de biomassa) e, iii) perceber se a recuperação do solo no fim de cada ciclo anual de tratamentos fitossanitários é possível. Para o primeiro objetivo, uma experiência laboratorial foi conduzida no qual solo artificial foi contaminado com concentrações crescentes de GLI ao qual se adicionou sementes de *M. sativa*. O crescimento das raízes e parte aérea foi afetado, sendo esse efeito acompanhado por um aumento da peroxidação lipídica, sugerindo a ocorrência de stress oxidativo, e por uma ativação do sistema antioxidante, mostrando que solos contaminados por GBH podem representar risco à sobrevivência de plantas não-alvo nas áreas mais contaminadas. Para o segundo e terceiro objetivos, foram recolhidas amostras em duas vinhas localizadas na Região Demarcada do Douro em modo de produção integrado (Quinta dos Aciprestes e Quinta do Casal da Granja) ao longo do ano de 2018 (fevereiro, abril e junho) e janeiro de 2019. Para a avaliação da qualidade do solo, foram analisados parâmetros físico-químico (por exemplo: pH; condutividade elétrica; matéria orgânica,

concentração pseudo-total de elementos maioritários e vestigiais e PF orgânicos sintéticos) e biológicos (atividade enzimática da desidrogenase, arilsulfatase, fosfatase e mineralização do azoto). Finalmente, para entender se a aplicação de PF afetou as funções do solo, ensaios ecotoxicológicos foram realizados com organismos terrestres (*Eisenia fetida* e *Medicago sativa*) e aquáticos (*Aliivibrio fischeri*, *Raphidocelis subcapitata* e *Lemna minor*). Os resultados mostraram que os efeitos observados para alguns parâmetros não podem ser atribuídos exclusivamente à aplicação de PF, uma vez que as propriedades físicas e químicas do solo também parecem contribuir para esses efeitos. No entanto, ainda que os PF parecem afetar a qualidade do solo no momento da aplicação, o sistema parece ser capaz de recuperar desta aplicação com o tempo.

Palavras-Chave: alfafa; ensaios ecotoxicológicos; espécies reativas de oxigénio; fungicidas; glifosato; herbicidas; stress oxidativo; modo de produção integrado; região demarcada do Douro; sistema antioxidante

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List of Abbreviations

AOX	Antioxidant
AMPA	Aminomethylphosphonic acid
AP	Acid phosphatase
Aryl	Arylsulfatase
CTL	Control
DDR	Douro Demarcated Region
DHA	Dehydrogenase activity
EC	Electric conductivity
FAO	Food and Agriculture Organization of the United Nations
GBH	GLY-based herbicide
GC-MS	Gas Chromatography Mass Spectrometry
GCC	Green cover crops
GLY	Glyphosate
GS	Glutamine synthetase
H_2O_2	Hydrogen peroxide
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
IPM	Integrated production mode
LC-MS	Liquid Chromatography Mass Spectrometry
LP	Lipid peroxidation
MDA	Malondialdehyde
N	Nitrogen
OECD	Organization for Economic Co-operation and Development
OM	Organic matter
PPPs	Plant protection products
Pro	Proline
QA	Quinta dos Aciprestes
QG	Quinta do Casal da Granja
RCV	Real Companhia Velha
ROS	Reactive oxygen species
TAC	Total antioxidant capacity
TPC	Total phenol content

1 Introduction

1.1 Introduction

Currently, the world is facing serious problems that must be addressed and resolved urgently to safeguard livelihoods and the economic and environmental sustainability of human societies. One of the most worrying issues is the population increase that, according to the Food and Agriculture Organization, will reach 9.15 billion in 2050; followed by climate change that severely affects the food production worldwide (FAO, 2009b). In line with these problems, agriculture faces multiple challenges, such as the growing market demand for food and animal feed, the adoption of more efficient and sustainable production methods, the capability to adapt and contribute to the mitigation of climate change, as well as the need to preserve natural habitats and maintain biodiversity (FAO, 2009a). In order to face these challenges, new technologies need to be developed to produce more from less land and resources and with more specialized human labor.

Agriculture is a very old activity that has been progressing especially over the last decades since it greatly contributes to the world economy and the sustainability of the population. In the specific case of viticulture, even though it does not contribute directly to the demand of food, in Portugal, it represents a significant percentage of the national agricultural sector, contributing greatly to the national economy (GPP, 2018). Moreover, the number of employees, in 2016, reached to 9.538 workers (AICEP Portugal Global, 2018). According to ViniPortugal, the total wine exports in 2019 exceeded 820 million euros (+ 20 million euros than the previous year), representing an increase of 2.5% compared to 2018 (ViniPortugal, 2019). Regarding the wine production, in 2019 there was 189,988 hectares of vines implemented being 29.1% of these located in Trás-os-Montes/ Douro and Porto region (Instituto da Vinha e Vinho I.P., 2020). Moreover, between 2019 and 2020 the Douro Region was responsible for 26% of the national wine production (Instituto da Vinha e Vinho I.P., 2020), much higher than other regions, demonstrating the importance that Douro has to the national economy. However, the importance of the viticulture from the Douro Demarcated Region (DDR) is not only attributed to its direct contribution to the economy but also to its unique landscapes that have a strong input in the tourism sector. However, since the European and International markets are very competitive, especially in the wine sector, there is a demand for a higher sustainability in the farming system, that must be attained without reducing the competitiveness of national wines. Thus, the need to increasingly implement sustainable management practices in terms of soil functioning arises, since a decline in soil quality will have a marked impact on vine growth and grape quality in a near future.

Soil can be defined as the top layer of the earth's crust, formed by mineral particles, organic matter, water, air and living organisms which interact through physical, chemical and biological processes (Jeffery et al., 2010). It is a unique environment that combines solid, liquid, and gaseous phases to form a three-dimensional matrix and, since it cannot renew rapidly, it is a non-renewable resource. Soils are responsible for many important services and functions in ecosystem processes. The ecosystem services such as soil formation, primary production, nutrient cycling, water and climate regulation, provision of food, fibers, and genetic resources, surface stability and refugia are supported by numerous soil functions, such as, carbon (C) storage, organic matter formation, storage and mineralization, mechanical support for plants, water storage, filtering and buffering, habitat provision for the soil organisms and anchoring support for human structures (FAO & ITPS, 2015). In this context, according to Doran and Parkin (1994), soil quality can be defined as *"the capacity of a soil to function within ecosystem boundaries to sustain biological productivity, maintain environmental quality and promote plant and animal health"*. In the specific case of viticulture, on which this project will focus, soil quality can be defined as the soil's capacity to support vine growth while reducing the negative effects on the environment (Riches et al., 2013). However, soil quality and its functions can be compromised by anthropogenic pressures within which agriculture is probably one of the activities with the greatest weight.

Agriculture has serious impacts on the soil, and some of the agricultural practices increase the soil's vulnerability to degradation processes like erosion, acidification, salinization and soil structure decline as well as contamination (Riches et al., 2013; Slavich, 2001). Conventional agricultural practices resort to the application of synthetic fertilizers and pesticides (Reganold, 1988) also known as Plant Protection Products (PPPs), since their use improves productivity, protects from crop loss or yield reduction, and controls disease vectors or agents. However, their use brings several problems namely, direct impacts on the environment, as well as both direct and indirect impacts on human health, through contamination of food, air, water and soil, and negative effects on non-targeted vegetation and organisms (Aktar et al., 2009). Moreover, some agricultural practices, such as PPPs application, are believed to cause negative impacts on the soil's ability to support agricultural production in the long term and adversely affect soil properties and functions (Riches et al., 2013). However, the toxicity and behavior that these PPPs will have in the environment depends upon their physical and chemical properties as well as the different processes that occur in soils such as sorption/desorption, volatilization, chemical and biological degradation, uptake by organisms and leaching (Arias-Estévez et al., 2008; Komárek et al., 2010).

As for viticulture, this practice is also known for the use of high amounts of inorganic and synthetic organic pesticides (Komárek et al., 2010, 2008; Patinha et al., 2017), especially copper-based formulations, in order to control vine diseases such as downy mildew caused by *Plasmopara viticola* (Komárek et al., 2010, 2008). This can lead to an accumulation of chemical substances on the soil, especially an increase of copper (Cu) concentrations (Komárek et al., 2008; Miguéns et al., 2007; Patinha et al., 2017) which can affect the phenology, growth and reproduction of some vineyards ruderal plant species (Brun et al., 2003), adversely affect soil organisms (Dumestre et al., 1999; Reinecke et al., 2008), and potentially contaminate the groundwater and adjacent water bodies (Komárek et al., 2008). The Douro region is of special concern regarding PPPs contamination since this area is characterized by vines implemented on steep slopes which contributes to soil erosion and, most importantly, to the runoff of these products (Patinha et al., 2017).

In order to minimize the negative impacts, related to conventional agricultural practices, other production modes have emerged. Integrated production mode (IPM) is one of them and has been increasing over the years since it is based on the rational management of natural resources with the ultimate goal of a more sustainable agriculture (Ministério da Agricultura do Desenvolvimento Rural e das Pescas, 2009). In Portugal, in 2017 there was 14,397 farmers that practiced IPM in 866 thousand hectares, being 7% of these attributed to vineyards[1]. IPM follows several principles such as: crop rotation to promote soil structure and fertility and reduce pesticide application, as it prevents the establishment of pathogens; minimum soil cultivation resulting in reduced soil erosion and nitrogen volatilization; targeted application of nutrients that reduces the amount of chemical application, improving the environment; rational use of pesticides; use of tillage systems that favors the natural control of pests and soil structure; and promotion of biodiversity (Morris & Winter, 1999). Particularly, the conservation and enhancement of biodiversity in cropping systems, both above and below ground biodiversity, are a priority. In the case of viticulture, two key factors have been identified as essential to improve the functional biodiversity: the implementation of ecological infrastructures conservation actions, such as the use of green cover crops in vineyards, and a sustainable use of PPPs.

Green cover crops (GCC) are non-agricultural crops used in viticulture specifically for covering the ground protecting the soil from erosion and loss of water and nutrients through leaching and runoff (Reeves, 1994), improving soil physical conditions and the environment. GCC use has other advantages such as pest control since it increases the

[1] https://www.dgadr.gov.pt/sustentavel/producao-integrada/controlo-e-certificacao-e-rotulagem

abundance of natural enemies of pests; improves soil fertility through the increase of soil nitrogen and organic matter; improves soil structure and water holding capacity and the establishment of beneficial microorganisms (Stefanucci et al., 2018). However, the benefits of GCC may be compromised by the application of PPPs that, even though are not directly used in cover plant species, they can still reach them through runoff or wind dispersion in case of spraying. Thus, the effects that PPPs application has on cover plants may jeopardize the balance of the ecosystem in which they are inserted, being necessary to study the scope of its action on these non-target species. This is especially important in the case of herbicides, such as glyphosate (GLY), a non-selective herbicide commonly used in vineyards to control weeds (Duke & Powles, 2008). Despite being a low-cost and efficient herbicide, the use of GLY has been associated with health (Nicolopoulou-Stamati et al., 2016; Samsel & Seneff, 2013; Swanson et al., 2014; Vazquez et al., 2017) and environmental problems since traces of it have been found in soils and groundwater (Aparicio et al., 2013; Battaglin et al., 2014; Lupi et al., 2015; Scribner et al., 2007; Silva et al., 2019, 2018) affecting both aquatic and terrestrial organisms (Van Bruggen et al., 2018). In the specific case of Portuguese vineyard soils, according to a study conducted by Silva et al. (2018), those were the soils with the highest concentration of GLY comparing with other European countries, reaching concentrations of 2.05 mg kg^{-1}. Thus, it is of upmost importance to understand the adverse effects that may result from this herbicide application to the vineyard soils and non-target species such as cover crops.

Since the use of PPPs is always needed despite the production mode, their sustainable use is another path to increase the soil's functional biodiversity in IPM. This can be achieved by several ways: implementing decision support systems (e.g. VitiMeteo, Vite.net) to control diseases development; selecting low toxicity PPPs that will contribute to the preservation and maintenance of natural enemies in the vineyard; mating disruption to control pests and reduce the use of conventional PPPs; microbial biocontrol agents such as the dissemination of natural enemies; and by using active ingredients of natural origin, a field that has been developing through the years (Stefanucci et al., 2018). However, the sustainable use of PPPs is not always achieved. This is mainly due to the fact that most studies related to the use of PPPs and their effects are performed in laboratory conditions and may not represent the reality of the field (Soares et al., 2019). On the other hand, the public opinion considers the use of PPPs as harmful, this being a good incentive for their sustainable use. For these reasons, there is an urgent need to monitor the impacts of PPPs application on the quality of the vineyard soils to assess if PPPs are being used in a sustainable way.

To assess soil quality, in terms of soil functioning and taking into consideration the different agricultural practices, physical, chemical, biochemical and biological indicators are usually measured (Andrews et al., 2004). However, since the soil matrix is a heterogeneous mixture of abiotic and biotic components, evaluating its quality can be quite complex. Typical physical and chemical indicators include texture, pH (in water and KCl), total content of organic matter and electric conductivity. Usually, some physical and chemical indicators are more correlated with geology being less responsive to changes induced by soil management practices. The biochemical and biological indicators, however, are more dependent on these practices being more sensitive and quickly react to environmental alterations (Antunes et al., 2011). In the particular case of soil enzymes, they catalyze a series of important reactions that are necessary for microorganisms and for the stabilization of soil structure since they play a very important role in the decomposition of organic matter and in the nutrient's cycling (Makoi & Ndakidemi, 2008). In addition, the alteration of the enzymatic activity of the soil allows the detection of the presence of toxic substances (Antunes et al., 2011), such as the contamination of the soil by PPPs used in IPM. Within the biological parameters, soil ecotoxicity can also be an important indicator of soil quality. Ecotoxicology studies the distribution, behavior, and bioavailability of contaminants on the environment and their effects on biota, natural communities and ecosystem functions (van Gestel, 2012). Thus, the ecotoxicological assays provide information about the presence of toxic elements and compounds in the soil as well as their impact on the soil biota community (Jensen & Mesman, 2006). In addition, through these tests it is possible to understand whether soil functions are altered or not with the application of PPPs (Ritz et al., 2009). The indicators mentioned above are often interconnected as noted by Dieckow et al. (2009) and Sugihara et al. (2010) by finding a relation between soil texture and microbial activity and organic matter and organic carbon content. Thus, it is necessary to interpret the relevance of soil biological indicators with the soil physical and chemical attributes and their ecological relevance (Doran & Parkin, 1997).

In this context, the three main objectives of this work were to assess the effects of soil contamination by a GLY-based herbicide (GBH) on the growth and redox homeostasis of a cover plant species, evaluate whether the application of PPPs in the DDR vineyards compromises soil quality and understand if the soil recover at the end of each annual cycle of PPPs application. In particular, this work intended to answer the following questions: i) Is the development and growth performance of a cover crop species affected by a GBH? ii) Is GBH's toxicity mediated by the occurrence of oxidative stress? iii) How do PPPs behave in the soil over time? iv) Are soil functions affected by PPPs? Are there any differences between sampling periods? With these questions we

intended to evaluate the following hypotheses: the use of GBH does not affect the growth and development of cover plant species, the application of PPPs does not affect soil functions and the soil functions do not recover between cycles of application of PPPs.

In order to achieve the three main objectives, two different studies, one experimental and one in the field, were conducted and presented in two different chapters, after this general introduction. Chapter two is entitled “Effects of Plant Protection Products on Cover Plants”, in this chapter, artificial soil was spiked with increasing concentrations of GLY in order to understand how this herbicide affected the growth and redox homeostasis of a cover plant *Medicago sativa*. In chapter three, soil samples from DDR vineyards were collected in different times of the year to verify if the application of PPPs affected soil quality and to understand if the soil can recover at the end of each annual cycle of PPPs application. Thus, chapter three has as title “Effects of Plant Protection Products on Vineyard Soils”. A final chapter with conclusions is also presented.

2 Effects of Plant Protection Products on Cover Plants

Fernandes, B.; Soares, C.; Braga, C.; Rebotim, A.; Ferreira, R.; Ferreira, J.; Fidalgo, F.; Pereira, R.; Cachada, A. Ecotoxicological Assessment of a Glyphosate-Based Herbicide in Cover Plants: *Medicago sativa* L. as a Model Species. Appl. Sci. 2020, 10, 5098.

2.1 Introduction

Plant protection products, also referred as pesticides, are widely used in agriculture in order to improve productivity, prevent crops' loss or yield reduction, and control disease vectors or agents. However, it is known that only a small portion of the applied pesticides reach the target pests, while the remainder will end up in soil or will have the potential to move to other environmental compartments, including ground and surface water (Duke, 2017; Pimentel, 1995). Nevertheless, the mobility of these contaminants in the environment depends on several biotic and abiotic variables, and of their physical-chemical properties. Thus, depending on the persistence of each substance, soil contamination can occur, thereby affecting soil quality, compromising its ability to perform its functions and leading to an irreversible degradation of this non-renewable resource (Aktar et al., 2009; Imfeld & Vuilleumier, 2012; Mahmood et al., 2016; Prashar & Shah, 2016; Silva et al., 2019). For this reason, concerns about the use of pesticides are increasing, and the most controversial at the moment is probably glyphosate (GLY), a post-emergence systemic herbicide of broad spectrum (non-selective). Applied to the foliage of weeds, GLY is absorbed by the leaves and is rapidly translocated in the plants through the phloem, particularly accumulating in meristems (root and shoot apex). Right after its discovery in the '70s of the last century, GLY quickly became the most applied herbicide worldwide and in 2014 the volume applied was sufficient to treat between 22 and 30% of globally cultivated cropland (Benbrook, 2016). Despite its great efficiency, several concerns about this herbicide were recently raised, related to the divergence between scientific studies regarding its toxicity to non-target organisms (Pochron et al., 2020; Van Bruggen et al., 2018). Another factor that may turn difficult to evaluate the real impacts of GLY on the environment is that GLY commercial formulations not only contain GLY, but also substances such as polyethoxylated amine (POEA) surfactants (Mesnage, Benbrook, & Antoniou, 2019). It is known that the first generation of POEA surfactants present in Roundup® were markedly more toxic than GLY, but since the mid-1990s, these compounds were progressively replaced by other POEA surfactants, ethoxylated etheramines, which exhibit lower non-target toxic effects (Mesnage et al., 2019). However, the composition of non-active ingredients in GBH is not fully known, and while a recent study pointed for a lower toxicity for earthworms of the GBH compared with the a.i. itself, (Pochron et al., 2020), other study concluded the opposite regarding *Dimorphandra wilsonii* seed germination (Gomes et al., 2017). Thus, GLY can be considered an old pesticide, but an emergent problem.

In areas in which high extensions of land are dedicated to intensive agriculture, the dispersion of GLY in the environment can be a serious problem of diffuse contamination,

particularly due to its tendency to adsorb to solid particles (Aparicio et al., 2013; Bento et al., 2017). Depending on climactic conditions (especially temperature and humidity), the removal of GLY from soils can be reduced, resulting in its accumulation (Bento et al., 2016). This accumulation and dispersion through the environment, due to its non-selectivity (Herrmann & Weaver, 1999; Zabalza et al., 2017), can cause damage to plants that are not targeted, affecting a great number of species that account both directly and indirectly for soil biodiversity. From the available data, it was suggested that GLY negative effects on plant growth and development, substantially exceed the effects triggered by its mode of action as it can induce several metabolic and physiological disorders, favoring the occurrence of oxidative stress as an indirect consequence (Gomes et al., 2014). Indeed, when plants are exposed to stress factors such as soil contamination, oxidative stress occurs due to an overproduction of reactive oxygen species (ROS) (Choudhury et al., 2013; Soares et al., 2019). Therefore, given their higher sensibility, ROS along with oxidative stress parameters (e.g. lipid peroxidation), can be used as exposure biomarkers allowing an early warning and sensitive evaluation of plants physiological status, representing a potential tool to phytotoxicity studies (Soares et al., 2016). Although ROS are important signaling factors, high levels of these compounds can easily become phytotoxic, damaging proteins, lipids, carbohydrates, and nucleic acids. By influencing the cellular gene expression pattern, ROS are involved in many processes such as growth, cell cycle, abiotic stress responses, pathogen defense and systemic signaling and development. Thus, in order to maintain the redox homeostasis of the cell, plants possess a powerful antioxidant (AOX) system, composed of both enzymatic and non-enzymatic mechanisms (Gill & Tuteja, 2010). It is the joint action of these players that prevent the occurrence of redox disorders in the cell, by directly neutralizing the toxic effects of ROS and/or by preventing their overaccumulation. However, depending on the plant species, the magnitude of stress and the exposure period, the AOX system may not be able to efficiently counteract ROS-induced toxicity, leading to the establishment of an oxidative stress condition (Soares et al., 2019).

One group of plants that is particularly exposed to GLY contamination is cover plants, since they can be sown few months after the herbicide application, during off-season. In crops such as vines, they can be sown between the lines and left as a green cover. They are of extreme importance to the management of soil erosion, fertility and quality as well as crop yield (Büchi et al., 2018; Wittwer et al., 2017). Indeed, the European Commission established that the maintenance of permanent grassland areas is one of the actions that each EU country and farmers must put in place, if they want to be rewarded for the protection of natural resources (European Commission, 2015). Thus, by affecting cover plants, GLY may jeopardize the balance of the ecosystem in which

they are inserted. An example of a cover plant is *Medicago sativa* L., commonly known as alfalfa, a perennial leguminous, belonging to the family Fabaceae and subfamily Faboideae, well known by its ability to improve both soils' structure and biochemical activity (Hamdi et al., 2012). This cover crop has the potential to establish symbiotic relations with N_2-fixing bacteria thus increasing its growth and development, while contributing for the enrichment of soils with nitrogen compounds (Hassouna et al., 1994; Zhu et al., 2016).

Since little is known about the potential phytotoxicity of GLY contaminated soil, particularly in non-target species, the aim of this work is to unravel the effects of soil contamination by this herbicide on the growth and redox homeostasis of a cover plant species, *Medicago sativa*. By combining biometrical and biochemical approaches, this study will focus not only on the effects of a GBH on the development and growth performance of *M. sativa*, but also on the assessment that whether its toxicity is mediated by the occurrence of oxidative stress.

2.2 Materials and Methods

2.2.1 Preparation of the artificial soil

The substrate used in this work consisted in an artificial soil composed of 70% (m/m) sand, 20% (m/m) kaolin and 10% (m/m) peat (OECD, 1984). The pH_{KCl} of the soil (1:5 m/v) was adjusted to 6.0 ± 0.5 by the addition of calcium carbonate ($CaCO_3$), whenever necessary.

2.2.2 Glyphosate (GLY) concentrations tested

The herbicide Roundup UltraMax® (Bayer, Germany), acquired from a local supplier, was used in this study. From the commercial formulation (360 g L^{-1} GLY as potassium salt), a stock solution was prepared and a series of sequential doses of GBH was applied, ranging from 0 to 40 mg kg^{-1} of the active ingredient (a.i.), with a dilution factor of 1.5, giving rise to the following concentrations: 40; 27; 18; 12; 8.0 mg kg^{-1}, which were tested together with a GBH-free control.

2.2.3 Plant material and growth conditions

The seedling emergence and seedling growth test, performed according to the OECD protocol for terrestrial plants (OECD, 2006a), was carried out in plastic pots containing 200 g of artificial soil, to which the solutions with the desired GLY concentrations were added. Maintenance of soil moisture was ensured by the presence of a pot with distilled

water placed at the base of the soil pots with soil, and by using a cotton rope to ensure the capillarity rise of the water. Twenty seeds of *Medicago sativa* [var. Dimitra, acquired from Flora Lusitana Lda (Cantanhede, Portugal)] were placed in each pot, after sterilization with 70% (v/v) ethanol (7 min) and 20% (v/v) commercial bleach (5% active chloride; 7 min), followed by washing with deionized water. To ensure the availability of nutrients, a commercial fertilizer (EcoGrow, NPK 3-6-7) was added at the start of the test. A negative control (CTL; absence of contaminant) was also prepared, obtaining a total of 24 pots (4 replicates for each treatment). The assay began when 50% of the seeds from the CTL germinated. In each pot, only 8 plants were kept, avoiding intraspecific competition. The plants germinated and grew in a growth chamber with controlled temperature (21 °C), photoperiod (16 h light/8 h dark) and photosynthetically active radiation (120 µmol m^{-2} s^{-1}). After 21 days of growth, plants from each replicate were collected, used for the estimation of biometric parameters and then, shoots were frozen in liquid nitrogen and stored at -80 °C until analyses.

2.2.4 Analysis of biometric indicators

The biometric analysis was performed as described in the OCDE protocol for seedling emergence and seedling growth test (OECD, 2006a). Eight plants from each replicate of every experimental group were used. After root and shoot separation, root length, and shoot height were measured, and the fresh mass of roots and shoots were registered.

2.2.5 Determination of physiological endpoints

Total chlorophylls (a + b) and carotenoids were extracted in 80% (v/v) acetone and quantified by spectrophotometry as described by Lichtenthaler (1987). The absorbance at 470, 647, and 663 nm was recorded, and the results obtained were expressed in mg g^{-1} fresh weigh (fw).

Total soluble protein content and glutamine synthetase (GS; EC 6.3.1.2) were extracted by homogenizing, on ice, frozen shoot samples in an extraction buffer, followed by a centrifugation at 4 °C for 20 min and 15 000 *g*. Afterwards, extracts were used to quantify the total soluble protein (Bradford, 1976) and to determine GS activity by the transferase assay (Ferguson & Sims, 1971) by recording the absorbance at 500 nm. GS activity was calculated and expressed as nkat mg^{-1} protein.

2.2.6 Quantification of oxidative stress biomarkers

The assessment of lipid peroxidation (LP) was performed as described by Heath and Packer (1968), by the quantification of malondialdehyde (MDA). Briefly, plant samples

were homogenized in 0.1% (w/v) trichloroacetic acid (TCA) and subsequently centrifuged (5 min; 10 000 *g*). Afterwards, the extracts were incubated with a mixture of 0.5% (w/v) thiobarbituric acid (TBA) and 20% (w/v) TCA for 30 min at 95 °C. At the end, the absorbance of each sample was read at 532 and 600 nm. After this step, the absorbance values of 532 nm were subtracted to the ones obtained at 600 nm to eliminate the effects of unspecific turbidity. The molar extinction coefficient (ε = 155 mM^{-1} cm^{-1}) was used to calculate MDA levels and the results were expressed as nmol g^{-1} fw.

The determination of hydrogen peroxide (H_2O_2) was performed according to the procedure described by Jana and Choudhuri (1981). Upon homogenization of shoot aliquots in 0.1% (w/v) TCA and centrifugation (6 000 *g*; 25 min), the obtained plant extracts were combined with a mixture containing 0.1% (w/v) $TiSO_4$ in 20% (v/v) H_2SO_4. Finally, the absorbance at 410 nm of each sample was recorded and the H_2O_2 levels were determined using the molar extinction coefficient of 0.28 μM^{-1} cm^{-1}. Results were expressed in nmol g^{-1} fw.

2.2.7 Analysis of the AOX response

In order to determine the total antioxidant capacity (TAC) and the total phenolics, the procedure described by Zafar et al. (2016) was followed. Firstly, frozen shoot samples were extracted in 80% (v/v) methanol followed by a centrifugation at 2500 *g*, for 10 min. Regarding TAC, upon dilution of the extracts (1:5), these were mixed with a reaction solution (0.6 M H_2SO_4, 4 mM ammonium molybdate and 28 mM sodium phosphate), incubated at 95 °C for 90 min, and cooled on ice. After that, the absorbance was read at 695 nm. TAC levels were obtained from a calibration curve obtained with dilutions of a standard solution of ascorbic acid (AsA) and the results expressed in mg equivalents of AsA g^{-1} fw. Concerning phenolics, their quantification was performed by a colorimetric assay using the Folin-Ciocalteu reagent. Absorbance was registered at 725 nm and total phenols concentrations were calculated from a calibration curve, prepared with dilutions of a gallic acid solution. The results were expressed in mg of gallic acid g^{-1} fw.

The extraction and quantification of proline (Pro) was performed as previously described by Bates et al. (1973), using the ninhydrin-based colorimetric assay. Samples were homogenized in 3% (w/v) sulphosalicylic acid and centrifuged (500 *g*; 10 min). Then, the extracts were incubated, under acid conditions, with a ninhydrin solution for 1 h at 96 °C. At the end, the absorbance of each sample was read at 520 nm and Pro content was obtained from a calibration curve obtained with known Pro concentrations, and the results were expressed as µg g^{-1} fw.

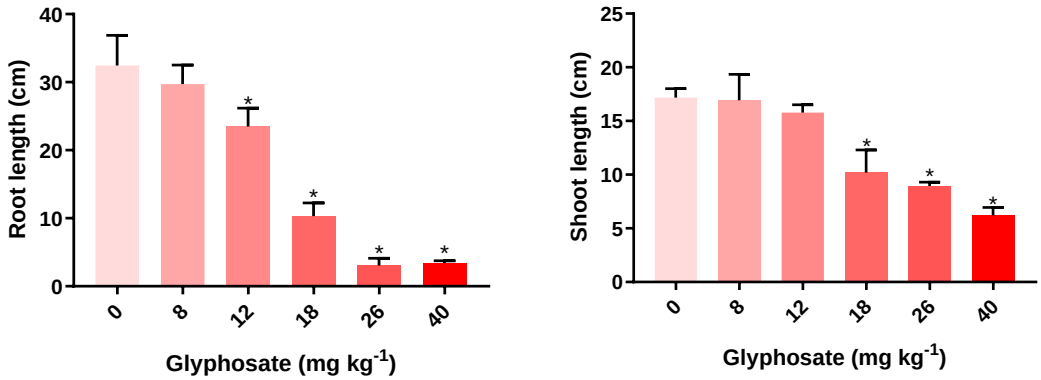
2.2.8 Statistical analyses

All endpoints were evaluated using, at least three replicates per treatment and results were expressed as mean ± standard deviation (SD). The effects of the herbicide on the parameters previously mentioned were evaluated using one-way ANOVA, after checking the homogeneity of variances by the Levene Test. Whenever $p \leq 0.05$, the *post-hoc* Dunnet's test was used to compare the mean of each group with the CTL. The EC_{50} (concentration of GLY expected to have an effect in 50% of test organisms) and the corresponding 95% confidence limits (95% CL) for the biometric parameters, were estimated with a non-linear least squares regression adjustment. All statistical procedures were performed in GraphPad Prism 8.

2.3 Results

2.3.1 Biometric parameters of *M. sativa*

As shown in Figures 2.1 and 2.2, the application of a GBH had a negative impact in both root and shoot length and biomass. By analyzing Figure 2.1a, it is possible to notice that there was a significant decrease in root length ($F(5, 16) = 106.8$; $p \leq 0.05$) for concentrations above the second lowest, with a monotonic dose-response relationship. Between 12 and 18 mg kg^{-1} of the a.i. there was a drastic reduction of root length: the inhibition values rose from 27% to 68% comparatively to the CTL group, the EC_{50} was estimated to be 16 mg kg^{-1} (95% CL:14-19).



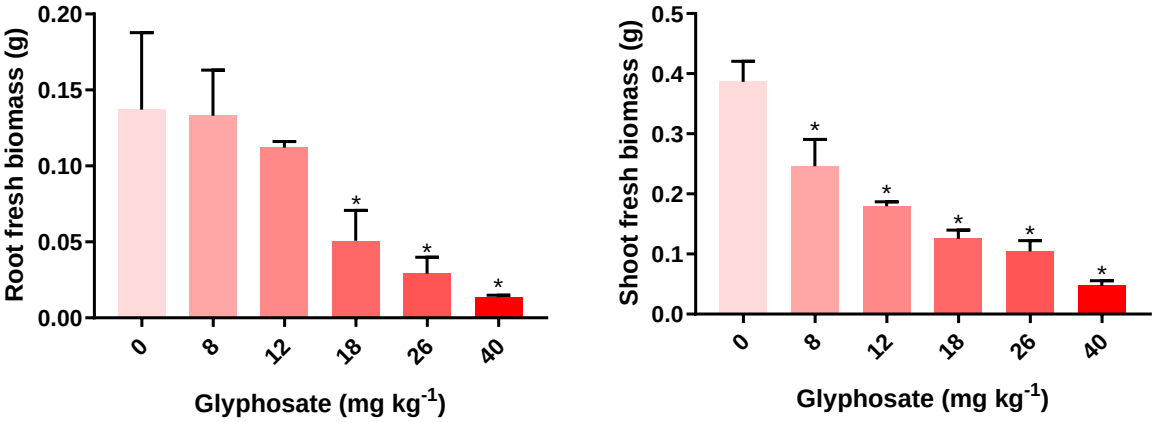
(a) (b)

Figure 2.1 Average root (a) and shoot (b) lengths of *M. sativa* plants, 21 days after exposure to different concentrations of GLY. Error bars correspond to the standard deviation. Statistically significant differences compared to the CTL (no GLY), considering $p \leq 0.05$, are marked with a * above bars.

Regarding shoot length, despite the observed decrease as the concentration increased, significant differences ($F(5, 16) = 36.21$; $p \leq 0.05$) were only recorded when plants were

exposed to the highest doses of GBH (18, 26 and 40 mg kg^{-1} of the a.i.), with inhibition values up to 64% in relation to the CTL. Nevertheless, a similar EC_{50} was estimated (16 mg kg^{-1} of the a.i.; 95% CL:14-22).

Regarding fresh biomass (Figure 2.2), both roots and shoots were affected by GBH exposure in a concentration-dependent manner. Despite both organs exhibited the same global trend, the results point towards a higher sensitivity of shoots when compared with roots. In fact, while in shoots, all concentrations are statistically different from the CTL ($F(5, 15) = 92.02$; $p \leq 0.05$), reaching inhibition values ranging from 36-88%, in roots biomass, significant differences ($F(5, 16) = 16.02$; $p \leq 0.05$) were only detected upon exposure to a.i. concentrations of 18, 26 and 40 mg kg^{-1}, with reductions of about 62, 79 and 90%, respectively. The highest effects observed in shoots are translated into differences in the EC_{50} values obtained. For root fresh biomass the estimated a.i. concentration was 15 mg kg^{-1} (95 % CL:12-22), whereas for the shoot fresh biomass it was 12 mg kg^{-1} (it was only possible to calculate the lower limit of the CL, which was 8.5).



(a) (b)

Figure 2.2 Average biomass of roots (a) and shoots (b) of *M. sativa* plants, 21 days after exposure to increased concentrations of GLY. Error bars correspond to the standard deviation. Statistically significant differences compared to the CTL, considering $p \leq 0.05$, are marked with a * above bars.

2.3.2 Physiological parameters on *M. sativa*

For the photosynthetic pigments, the behavior was similar for both total chlorophylls and carotenoids (Figures 2.3a and 2.3b, respectively), as no significant statistical differences were registered among treatments and the CTL: $F(5, 12) = 2.072$; $p > 0.05$ for total chlorophylls and $F(5, 8) = 2.920$; $p > 0.05$ for carotenoids.

GS levels (Figure 2.3c) showed a different pattern from that of the photosynthetic pigments. Comparatively to the CTL, all GBH concentrations induced a significant reduction in GS activity levels ($F(5, 12) = 7.851$; $p \leq 0.05$). As can be observed in Figure

2.3c, when plants were exposed to a.i. concentrations between 8 and 26 mg kg^{-1}, decreases of around 50% were found in comparison with the CTL. Curiously, upon exposure to the highest concentration, GS levels became closer to the ones registered for the CTL.

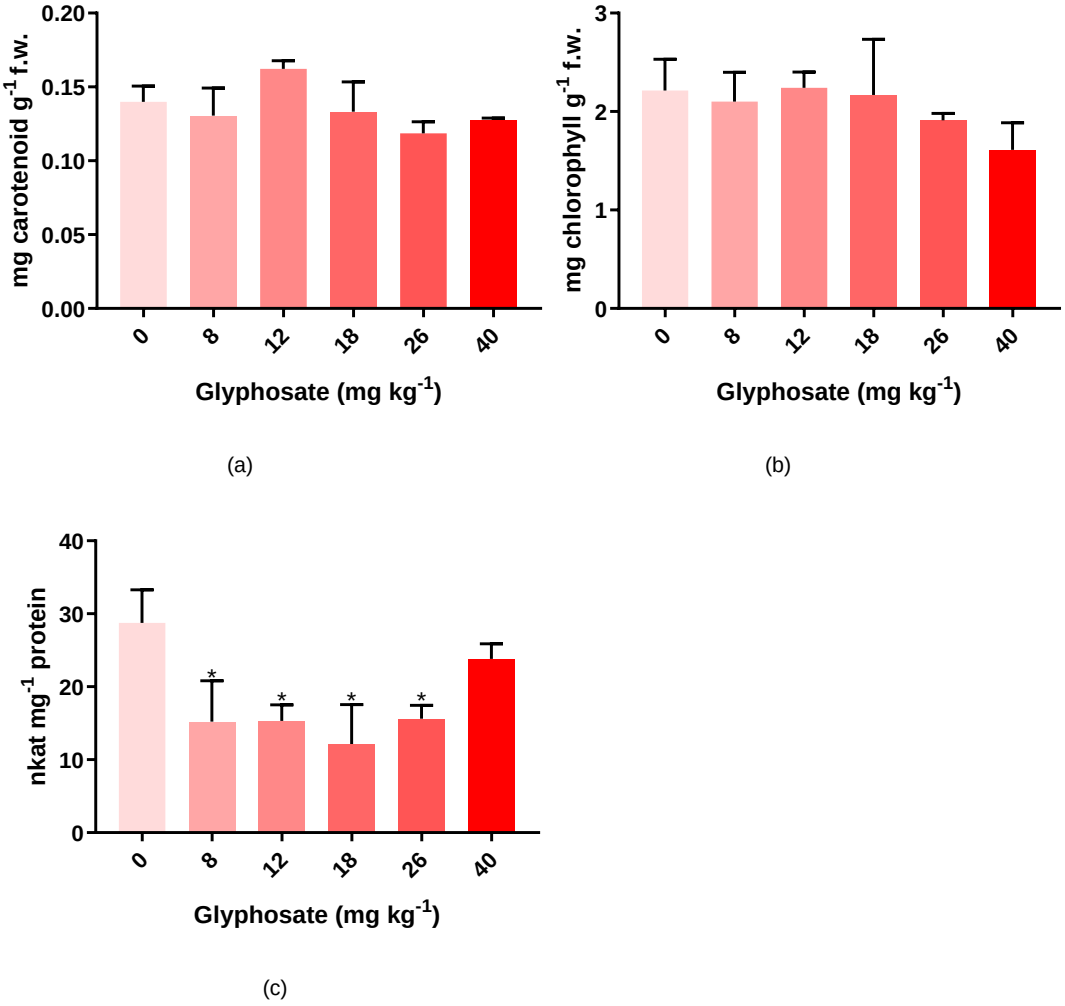
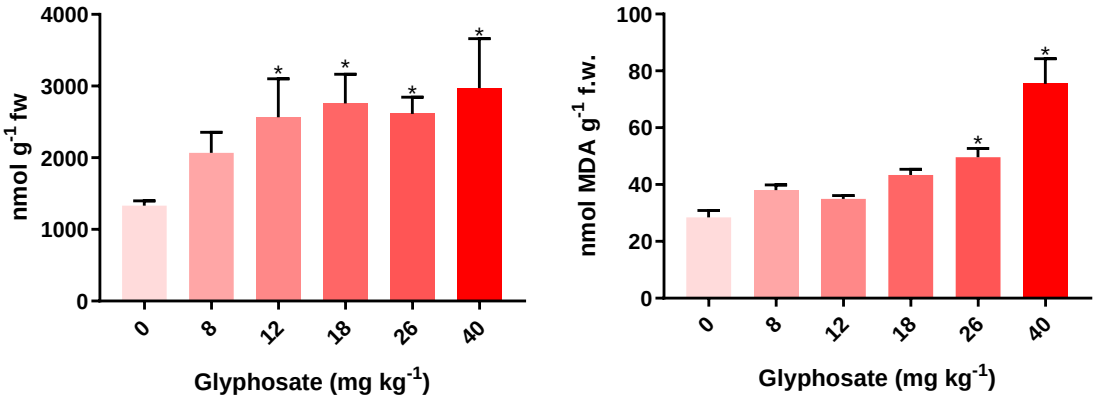


Figure 2.3 Average concentrations of carotenoid (a) and chlorophyll (b) and GS activity levels (c) in shoots of *M. sativa* plants 21 days after exposure to increased concentrations of GLY. Error bars correspond to the standard deviation. Statistically significant differences compared to the CTL, considering $p \leq 0.05$, are marked with a * above bars.

2.3.3 Oxidative stress biomarkers on *M. sativa*

The behavior of the analyzed oxidative stress biomarkers, H_2O_2 and LP, is shown in Figure 2.4. In general, H_2O_2 levels rose along with the increase of GBH concentration (Figure 2.4a). However, significant differences ($F(5, 11) = 6.294$; $p \leq 0.05$) were only observed for concentrations higher than 12 mg kg^{-1}, compared to the CTL. A similar behavior was also observed for LP with MDA levels increasing in a concentration-dependent manner (Figure 2.4b). Despite of this pattern, for LP, statistically significant differences from the CTL ($F(5, 30) = 13.37$; $p \leq 0.05$) were observed only at the highest a.i. concentrations (26 and 40 mg kg^{-1}).



(a) (b)

Figure 2.4 Average concentrations of H_2O_2 (a) and MDA (b) in shoots of *M. sativa* plants 21 days after exposure to increased concentrations of GLY. Error bars correspond to the standard deviation. Statistically significant differences compared to the CTL, considering $p \leq 0.05$, are marked with a * above bars.

The AOX response, evaluated by assessing the TAC, total phenol content (TPC) and Pro levels, of *M. sativa* exposed to Roundup UltraMax® is presented in Figure 5. Regarding TAC (Figure 2.5a), although a tendency for enhanced values as the concentration of the GBH goes up, statistical significant differences ($F(5, 14) = 3.468$; $p \leq 0.05$) were only found when plants were exposed to 40 mg kg^{-1} of a.i., with an increase of about 75% above the CTL. On the other hand, TPC (Figure 2.5b) was reduced upon exposure to increased concentrations of the GBH, especially in the highest dose (decreases up to 36%). Indeed, significant differences ($F(5, 13) = 7.802$; $p \leq 0.05$) comparing to the control were observed only for the higher concentration. Concerning Pro (Figure 2.5c), its content showed a similar pattern to that of TAC, with levels significantly higher ($F(5, 8) = 5{,}574$; $p \leq 0.05$) than the CTL (by threefold) only for the highest concentration of GLY.

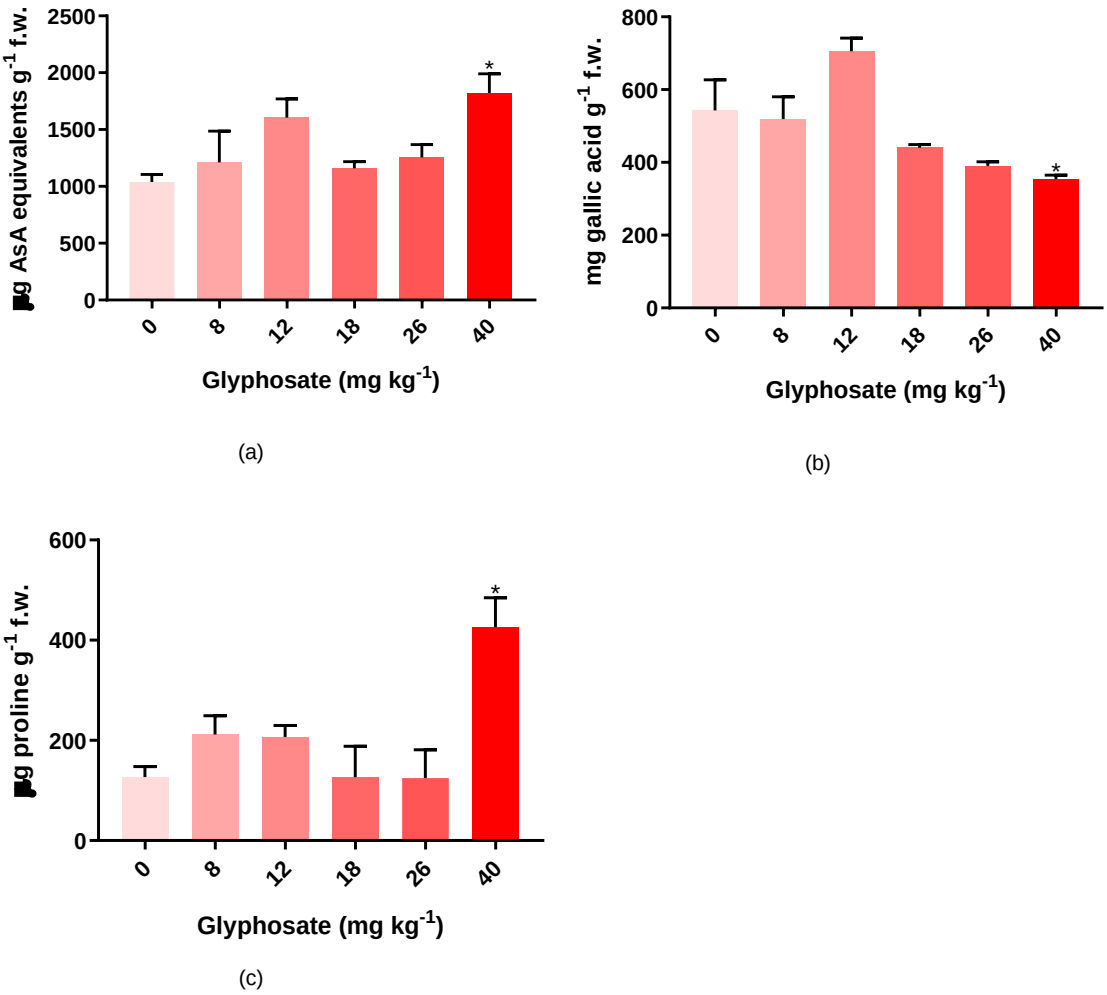


Figure 2.5 Effect of increased concentrations of GLY, on the AOX system of *M. sativa* shoots after 21 days of exposure. (a) TAC; (b) TPC; (c) Pro. Error bars correspond to the standard deviation. Statistically significant differences compared to the CTL, considering $p \leq 0.05$, are marked with a * above bars.

2.4 Discussion

Up to date, little is known regarding the phytotoxicity of GLY contaminated soils on non-target plants, including cover crop species, such as *M. sativa*. Although these plants are not intentionally treated with GLY, they can still be affected by its application through leaching, runoffs or even wind in case of spraying. Moreover, GLY strongly adsorbs to solid particles (Aparicio et al., 2013; Bento et al., 2017) and accumulates in soils (Bento et al., 2016), resulting in a serious problem of diffuse contamination. Indeed, several studies were conducted in order to determine GLY levels in soils around the world and despite many of them reported levels lower than 3 mg kg^{-1} for agricultural soils or soil located nearby agricultural areas in South America (Alonso et al., 2018; Aparicio et al., 2013; Primost et al., 2017; Soracco et al., 2018) and Europe (Grunewald et al., 2001a; Karanasios et al., 2018; Laitinen et al., 2006; Silva et al., 2019, 2018), other studies have

reported values of 5.0 mg kg^{-1} in soybean cultivated areas in Argentina (Peruzzo et al., 2008), reaching values as high as 40.6 mg kg^{-1} in olive groves from Greece (Karanasios et al., 2018) or even 608 mg kg^{-1} in a crop field from Mexico (Muñoz et al., 2019). Therefore, the main goal of the present study was to assess the effects of soil contamination by a GBH on the growth responses and redox homeostasis of alfalfa plants, at environmental relevant concentrations of the a.i. In fact, despite recent studies have been conducted to evaluate the effects of GLY application in non-target plants, most of these works applied GLY as foliar spray (Akbulut, 2014; Gomes et al., 2017; Gomes et al., 2015; Krenchinski et al., 2017; Radwan & Fayez, 2016; Singh et al. 2017; Singh et al. 2017) or as a supplement to the nutrient solution (De Campos Oliveira, 2016; de Freitas-Silva et al., 2017; Gomes et al., 2017; Mondal et al., 2017; Serra et al., 2015; Tong et al., 2017) rather than simulating soil contamination scenarios.

The present study showed that, after 21 days of exposure, Roundup UltraMax® severely repressed the growth of *M. sativa*, in a dose-dependent manner, inhibiting both organs' elongation and biomass production. Actually, given the already accentuated reduction of shoot fresh weight upon exposure to the lowest concentration tested (8 mg kg^{-1} of a.i.), it can be suggested that even lower concentrations would be capable of impairing plant growth. When GLY is absorbed by the plant, it is translocated through vascular tissues, namely by phloem, reaching active metabolite sites, such as root and shoot meristems, following the same pathway as photoassimilates (Gomes et al., 2014; Satchivi et al., 2000) which could explain the repression of shoot growth. The fact that GLY is an herbicide that inhibits an enzyme from the shikimate pathway, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19), can also explain the results obtained. EPSPS plays a role in the synthesis of the aromatic amino acids tryptophan, phenylalanine, and tyrosine that are crucial for the growth and survival of plants and which function as the precursors of many secondary metabolites such as pigments, auxins and lignin (Herrmann, 1995). As a result of the shikimic acid pathway being blocked, there will be an accumulation of shikimate in plant tissues which will lead to a deficit in important end products such as lignin, alkaloids, and flavonoids, and a reduction in CO_2 fixation and biomass production in a dose-dependent manner (Olesen & Cedergreen, 2010). The decrease in root and shoot length and biomass can also be due to the impact that GLY has on indole-3-acetic-acid (IAA) metabolism which is the main endogenous auxin in the plant as well as interfere with plant-water relationship (Clay & Griffin, 2000; Mondal et al., 2017; Soares, Pereira, et al., 2019). Another hypothesis that can explain these results is the fact that GLY can condition the absorption of several macro and micronutrients such as Ca, Mg, N, P, Fe, Zn among others as reviewed by Gomes et al. (2014).

Several studies were conducted in order to evaluate the phytotoxicity of GLY to non-target plants such as: *Pisum sativum* (GLY or GBH, applied directly to the seeds or supplemented to the nutrient solution) (Mondal et al., 2017; Singh et al., 2017); *Hordeum vulgare* (GBH supplemented to a mixture of perlite:vermiculite (1:2)) (Spormann et al., 2019); *Solanum lycopersicum* (GLY applied by foliar spray) (Singh et al., 2017); *Vigna radiata* (seeds treated with a GBH) (Basantani et al., 2011); *Fagopyrum esculentum* (GLY isopropylamine salt supplemented to the nutrient solution) (Debski et al., 2018); *Lemna minor* (GBH supplemented to the nutrient solution) (Sikorski et al., 2019)*; and Dimorphandra wilsonii* (seeds treated with a GBH or analytical grade glyphosate) (Gomes et al., 2017). Even though the experimental conditions of the previously mentioned studies were not similar to the present study, they all recorded a decrease in plant growth demonstrating the negative effect that both GLY and GBH have on biometric indicators. Concerning GBH-contaminated soils, a similar decrease was also observed in the work of Soares et al. (2019b), in which tomato plants grew in an artificial soil contaminated by increasing a.i. concentrations (0, 10, 20 and 30 mg kg^{-1}). Their results showed significant statistical differences even at 10 mg kg^{-1}, a concentration pretty much identical to the lowest dose tested in this study.

Photosynthesis, one of the main biochemical process occurring in photoautotrophic organisms, highly depends on light absorption by chlorophylls and carotenoids. The biosynthesis of these pigments as well as fatty acids or amino acids can be affected by GLY exposure (Fedtke & Duke, 2005). As GLY is an EPSPS competitive inhibitor, it blocks the shikimate pathway, thus compromising the biosynthesis of secondary metabolites, such as quinones, and photosynthetic pigments, all compounds involved in the photosynthetic metabolism (Dewick, 1986). Previous studies showed that GLY can impair plastoquinone synthesis, thereby contributing for a lower production of carotenoid precursors (Gomes et al., 2015). Regarding chlorophylls, both GLY and GBH can also directly inhibit its biosynthesis, by reducing δ-aminolevulinic acid (ALA) levels, or increase chlorophyll degradation as reported by several authors (Gomes et al., 2015; Huang et al., 2012; Kitchen et al., 1981; Mateos-Naranjo et al., 2009; Singh et al., 2017; Zobiole et al., 2011). Based on these results, it was expected to observe a significant decrease of the levels of both chlorophylls and carotenoids. Indeed, even a previous work conducted with the same plant species but grown in perlite and quartz sand (Muñoz-Rueda et al., 1986) reported that the foliar application of a GBH resulted in a reduction of the total photosynthetic pigments as the a.i. concentration increased. However, in the present study, the herbicide showed no effects on chlorophyll and carotenoid contents, despite the slightly lower contents observed when comparing to the control group (except for 12 mg kg^{-1}). Thus, these results suggest that, at the tested

doses, this herbicide did not negatively affect the photosynthetic pigments as also demonstrated in the study performed by Spormann et al. (2019) with a GLY concentration of 30 mg kg^{-1}, applied in the form of Roundup UltraMax® and using a mixture of perlite:vermiculite as substrate. As discussed by Spormann et al. (2019), these results could be explained by the lack of AMPA production in the artificial medium. Indeed, AMPA, the main metabolite formed upon GLY degradation, is considered as a potent phytotoxin, capable of competing with glycine and consequently inhibiting chlorophyll biosynthesis (Reddy et al., 2004; Serra et al., 2013). Thus, there are two hypotheses for the lack of negative effects due to GLY exposure on chlorophyll and carotenoid content i) the use of a standard artificial soil with low microbial activity not allowing enough AMPA production to cause negative effects on biosynthesis of these pigments, and/or ii) the mode of application of GLY, which, in this study, was added to the soil contrasting to the majority of works which provided GLY as foliar spray. However, and regarding the former hypothesis, this does not mean that an enhanced effect on a natural soil with a more diverse and functionally active soil microbial community would certainly be expected, as the degradation rates of both GLY and AMPA are still not well studied.

As important as photosynthesis, the mineral nutrition of plants highly contributes for a proper growth performance. However, the effect of GLY on plant mineral nutrition is yet to be fully understood (Zobiole et al., 2010). Up to now, no consensus has been reached on the influence that GLY may bring on nutrients uptake, since the studies conducted so far point towards different results: while several authors reported a negative effect of GBH on plant's nutrient uptake (Cakmak et al., 2009; Zobiole et al., 2012; Zobiole et al., 2011; Zobiole et al., 2010), other studies concluded that this application does not affect the mineral status of the plants (Bailey et al., 2002; Duke et al., 2012; Duke et al., 2012). As reviewed by Duke et al. (2012), these inconsistent results may be due to differences in the type of soil, climatic conditions, and/or GLY-resistant cultivars used. Aiming to assess the nutritional status of *M. sativa* under GLY exposure, the present study evaluated the activity of GS, an enzyme that is involved in the first step of ammonium ($NH4^+$) assimilation, not only that absorbed by roots, but also that generated from photorespiration, proteolysis and processes that are increased by several stresses (Gomes Silveira et al., 2003; Pageau et al., 2006). The results revealed that GS was dysregulated for almost all tested concentrations, indicating that, at least under the experimental conditions of the present work, GBH interfered with the nitrogen (N) metabolism. Based on these findings, the hypothesis that GLY conditioned the physiological uptake of mineral nutrients specially nitrogen (N), due to the formation of complexes making them unavailable for biological processes, arises (Zhong et al., 2018).

Concerning N uptake, once again, results from different studies, all of them using GBH, are contradictory with no effect in field studies (Bellaloui et al., 2006; Henry et al., 2011) and inconsistencies in greenhouse studies (Cakmak et al., 2009; Zobiole et al., 2010).

As previously reviewed by Gill and Tuteja (2010) and Soares et al. (2019), plant development can be severely affected by various abiotic stressors such as herbicide application, leading to an overproduction of ROS which in its turn will cause significant damage to cell structures ultimately resulting in oxidative stress. In order to verify the occurrence of oxidative stress, H_2O_2 levels and LP degree, as a mean to assess membrane damage, were evaluated. According to the results obtained, H_2O_2 accumulation was enhanced upon exposure to GBH, especially at levels of the a.i. higher than 12 mg kg^{-1}. However, when looking to LP results, MDA content was only increased in response to the two highest treatments (26 and 40 mg kg^{-1} of a.i.). Based on this behavior, one can suggest that ROS overproduction took place earlier than the observed membrane damage, being this possibly related to the dual role played by ROS in plant cells. Indeed, H_2O_2, as other ROS, can act as a signal molecule at low concentrations as it is involved in acclimation signaling leading to plant tolerance to various biotic and abiotic stress, becoming toxic above a certain threshold, capable of inducing programmed cell death (Quan et al., 2008). Therefore, it can be hypothesized that, at lower GLY concentrations, H_2O_2 was involved in signaling mechanisms (with no LP increase), while at the highest concentrations (26 and 40 mg kg^{-1} of the a.i.), H_2O_2 accumulation started to induce oxidative damage, which is reflected by the occurrence of LP.

The induction of oxidative stress by GLY is described as one of its indirect effects on plant physiology, either by the overproduction of ROS or by a depletion of defense mechanisms (Gomes et al., 2015). Although not so explored as in target and resistant species, the influence of this herbicide on the redox status of non-target plants, including crops, willow and aquatic plants (Akbulut, 2014; Gomes et al., 2017; Gomes & Juneau, 2016; Gomes et al., 2015; Moldes et al., 2008; Radwan & Fayez, 2016; Singh et al., 2017; Singh et al., 2017; Soares et al., 2019; Spormann et al., 2019; Zhong et al., 2018) is starting to gain attention. Corroborating the results of the present work, several studies reported an increase in H_2O_2 content and MDA levels in plants grown in GBH-contaminated solid substrate (Spormann et al., 2019), or when GLY or GBH was supplied in nutrient solutions (Gomes et al., 2017; Gomes & Juneau, 2016; Gomes et al., 2015; Singh et al., 2017), or applied as foliar spray (Akbulut, 2014; Radwan & Fayez, 2016; Singh, Singh, Singh, & Hussain, 2017). However, according to Moldes et al. (2008) and Soares et al. (2019b), the exposure of soybean and tomato plants to GBH did not induce a severe oxidative damage in leaves.

In order to defend themselves from oxidative damage caused by ROS, plants developed protective mechanisms by synthetizing enzymatic and non-enzymatic antioxidants (Gill & Tuteja, 2010). In the context of this work, TAC, TPC and Pro levels were measured to assess the involvement of the non-enzymatic component of the AOX system in limiting GLY-induced stress. The results showed an increase in TAC and Pro levels only at the highest a.i. concentration (40 mg kg^{-1}). Since TAC gives a general idea regarding the cell's AOX status (Pinto et al., 2019) and Pro acts as a strong AOX (Gill & Tuteja, 2010), the elevated TAC and Pro levels suggest that the AOX defense mechanisms were activated due to oxidative stress, but only at the highest concentrations of GLY. Thus, it can be hypothesized that *M. sativa* plants boosted the accumulation of Pro, along with other non-enzymatic players, to counteract the induced oxidative stress by this herbicide; however, bearing in mind that LP remained higher at the two highest concentrations, this response was not enough to counteract the harmful effects observed. Moreover, phenolic compounds, which are known to chelate metals, scavenge ROS and inhibit LP (Sharma et al., 2012), were negatively affected by the presence of the herbicide, since reduced levels of these specialized metabolites were found in treated plants. This effect probably arises as a consequence of GLY-induced impairment of the shikimate pathway, once phenolic compounds are formed through this biosynthetic process (Santos-Sánchez et al., 2019), and is in accordance with the results obtained for LP.

Up to now, some studies were conducted in order to evaluate the AOX defense mechanisms of plant species exposed to both GLY and GBH (Gomes et al., 2017; Sergiev et al., 2006; Singh et al., 2017; Soares et al., 2019; Spormann et al., 2019). These studies demonstrate that there is a dysregulation of the AOX defense system, with records of both increases and decreases of these mechanisms. Particularly, in the study of Soares et al. (2019), performed with GBH-contaminated soils, it was observed that this formulation stimulated the AOX defense mechanisms of tomato shoots, at concentrations of 20 and 30 mg kg^{-1} of the a.i.. This suggests that like other environmental stresses, the response to herbicide application depends on several factors such as the plant species, the concentration, and the mode of application. However, the results obtained in the present study are in line with those already published by other authors (Gomes et al., 2017; Singh et al., 2017; Soares et al., 2019; Spormann et al., 2019) indicating that the increase in Pro levels seems to be the most consistent signal of the activation of the AOX defense against GLY-induced stress, suggesting that this amino acid can be used as a biomarker of exposure to GLY.

3 Effects of Plant Protection Products on Vineyard Soils

3.1 Introduction

Plant Protection Products (PPPs) are composed of at least one active substance with the purpose of: protect plants against pests or diseases, either before or after harvest; affect the life processes of plants, for example, substances influencing their growth (excluding nutrients); preserve plant products; destroy or prevent growth of undesired plants[2]. Even though the use of PPPs protects crops, contributing to greater productivity and product quality, it poses serious risks not only for human health but also for the environment. So far, several problems associated with the use of PPPs have been described in literature, namely: air pollution due to PPPs spraying, wind erosion and tillage activities in agricultural soils; direct impacts on human health through the ingestion of food products that may contain traces of the PPPs used; adverse effects on non-target organisms such as the reduction of populations of beneficial organisms; contamination of water resources by leaching or mobilization of active ingredients and/or coadjutant compounds, leading to potential adverse effects in aquatic organisms; sub-lethal effects and elimination of non-target plants (Aktar et al., 2009; Carvalho, 2017; Damalas & Eleftherohorinos, 2011; Katra, 2020; Mahmood et al., 2016; Manea et al., 2017).

The use of PPPs is of special concern in vineyard areas, especially the ones located on steep slopes of narrow valleys such as the ones from Douro Demarcated Region (DDR), in the North of Portugal. This area is particularly sensitive to erosion processes and runoff which leads to the transport of pollutants, becoming a potential source of contamination to freshwater courses (Patinha et al., 2017). Viticulture is known for the use of large amounts of inorganic and synthetic organic PPPs which, allied with the intense vine cultivation can lead to an accumulation of these contaminants and its degradation products in the vineyard soils (Komárek et al., 2010; Patinha et al., 2017). Since the use of these products is associated with negative effects, there is an increasing effort for a more sustainable use. In this sense, integrated production mode (IPM), that has as principals the reduction of PPPs application and the increase of soil's biodiversity, among others, is seen as more sustainable alternative comparing with conventional production (Morris & Winter, 1999). Even though there are already several studies reporting PPPs residues in Portuguese vineyard soils as well as in other countries, there is no reference to the production mode of the targeted vineyards (Patinha et al., 2017; Pérez-Mayán et al., 2020; Rial-Otero et al., 2004; Silva et al., 2019).

Regarding the PPPs commonly found in vineyard soils, as reviewed by Komárek et al. (2010), synthetic organic fungicides such as procymidone, metalaxyl, fludioxonil,

[2] https://ec.europa.eu/food/plant/pesticides_en

cyprodinil and tebuconazole were found at higher concentrations in Spain. However, the authors recorded that the application time of the fungicides significantly influenced their concentrations. Silva et al. (2019) in a study on the distribution of pesticide residues in agricultural topsoils across the European Union (EU), found that the herbicide glyphosate (GLY) and its metabolite AMPA, the formerly used insecticide DDT and, the broad-spectrum fungicides boscalid, epoxiconazole an tebuconazole were the compounds most frequently found and at the highest concentrations. In the specific case of Portugal, the residues found in vineyard soil samples with the highest concentrations were AMPA, GLY and phthalimide (a metabolite of the fungicide folpet). In fact, Silva et al. (2018) in their study with the purpose of evaluating the distribution of GLY and AMPA in agricultural topsoils of the EU found that vineyard soils were those with the highest concentrations of GLY reaching concentrations of 2 mg kg^{-1} in Portuguese vineyard soils, the highest of other EU countries. According to Patinha et al. (2017), metalaxyl, boscalid and penconazole were the three out of the five fungicides analyzed that were present at higher concentrations in the vineyard soil samples. A concerning fact was the high levels of DDT, an insecticide banned in Portugal since 1988 (Ministério da Agricultura do Desenvolvimento Rural e das Pescas, 1988), that were found in the oldest vineyards (> 50 years). The same pattern was also observed for the total Cu content which ranged between 18 and 211 mg kg^{-1} being the highest concentrations also found in vineyards with more than 50 years. This Cu accumulation with vine age was also reported by Morgan and Taylor (2004), Rusjan et al. (2007) and Miotto et al. (2017) being mostly attributed to the use of Cu-based fungicides to prevent vine diseases such as downy mildew caused by *Plasmopara viticola*. Taking all of this in consideration, in addition to the accumulation of PPPs in the soil, another problem arises associated with its application, the cumulative and the mixture effect. Even though these products may not be present in the soil in high concentrations, their combination may cause harmful effects to the ecosystem (Uwizeyimana, Wang, Chen, & Khan, 2017; Yijun Yu et al., 2019). However, attributing these negative effects to PPPs combination is difficult.

PPPs behavior, mobility, bioavailability, persistence and toxicity are dependent on different processes occurring in soils such as sorption–desorption, volatilization, chemical and biological degradation, uptake by plants and leaching as well as from their physical and chemical properties such as vapor pressure, stability, solubility, pK_a (Arias-Estévez et al., 2008; Damalas & Eleftherohorinos, 2011). Besides this, the effects that PPPs may have in soils are also influenced by the climate conditions at the time of application (Uwizeyimana et al., 2017).

An important tool to evaluate the effects that single or PPPs' mixture may have on soils is the ecotoxicological assays. Even though some soil physical and chemical

parameters may give an indication of the impacts that PPPs application have on soil's quality, this assessment must be complemented with the assessment of the effects on soil biota, due to their higher sensitivity to biological processes and the capacity of organisms to detect and rapidly respond to contaminants that are available for uptake (Alves & Cardoso, 2016). Thus, the effects of PPPs application on soil functions, such as biomass production, habitat provision and retention of contaminants, can be assessed by ecotoxicological assays giving an indication of the soil quality (Ritz et al., 2009). So far, few studies (Wightwick et al., 2013) were conducted in vineyard soils aiming to understand how PPPs application affects soil functions, however, Eijsackers et al. (2005) and Brun et al. (2001) found negative effects on terrestrial organisms such as earthworms and plants, which could indicate that soil functions were compromised. In the specific case of DDR, as far as we know, there are no studies regarding the effects of PPPs application on soil functions. Most studies that already exist on the impact of PPPs application on soils are carried out in laboratory conditions and often with high doses, which are not representative of reality (Scheepers & Godderis, 2019).

Since there is no EU legislation for thresholds or quality standards for total or individual PPPs residues in soil, the evaluation of PPPs contamination, and the characterization of overall soil quality, should integrate several lines of evidence in order to achieve the target of sustainable food production. Thus, the two main objectives of the work described in this chapter was to assess if the application of PPPs is compromising the soil quality in vineyards from DDR and to have insights on the possible recovery of soils at the end of each annual cycle of phytopharmaceutical treatments. In particular, this work intended to answer the following questions: i) How do PPPs behave in the soil over time? ii) Are soil functions affected by PPPs? iii) Are there any differences between sampling periods, which took into account different annual applications? With these questions we intended to evaluate the following hypotheses: i) the application of PPPs does not affect soil functions and, ii) the soil functions do not recover between cycles of application of PPPs. Therefore, soil samples were collected in vineyards from two farms located in DDR, both under IPM. The sampling took place in four different periods, namely, during herbicide application, two months after herbicide application, during fungicide application and finally, before herbicide application for the next agricultural year. Soil’s physical and chemical characterization was made, and the levels of PPPs were determined. Some microbial parameters as well as ecotoxicological assays with terrestrial and aquatic organisms were also performed.

3.2 Materials and Methods

3.2.1 Study area

The study area is located on the Douro region, with vines implemented on its traditional steep slopes that, since 2001, is considered by UNESCO as a World Heritage Site due to its unique landscapes and cultural legacy[3], contributing to the tourism sector. This region extends along the Douro River and its tributaries in an extension of about 250,000 hectares and the geological formation is of schist-greywacke origin where soil is almost non-existent being mostly all man-made. It is characterized by an extremely rugged orography which is limited by mountains on the South and West and planar areas on North and East. In terms of climate, the DDR has very cold winters and very hot and dry summers with water scarcity and temperatures that are favorable to the full ripening of the grapes resulting in wines of great quality (Andresen, De Aguiar, & Curado, 2004).

For the purpose of this work, two plots on two different farms from "*Real Companhia Velha*", were selected, namely: Quinta dos Aciprestes and Quinta do Casal da Granja.

The Aciprestes farm (QA) is located on the left bank of Douro river (Figure 3.1), in the mouth of the Tua river, and extends for more than two kilometers. It is located between 100 and 300 meters in altitude with a 30-50% slope. The climate of this area is characteristically Mediterranean with two different seasons: wet and cold from October to April; dry and warm from May to September. The highest temperatures and the water deficit during the Summer result in high levels of dryness. The grape varieties in this farm are: "Tinta Barroca", "Touriga Franca", "Tinta Roriz", "Touriga Nacional", "Sousão" and "Tinta Amarela" (RCV, 2014).

Quinta do Casal da Granja (QG) is located on the right side of the Douro River (Figure 3.1), on Alijó plateau, and it has 170 hectares of vines, implanted on a slightly undulated landscape with an altitude between 520 and 640 meters and a slope of 15%, which is privileged for white wine production. The climate of this farm is characterized by high levels of rainfall, and it has colder winters (snowing with some regularity) and cooler summers, when comparing with Aciprestes farm. The maximum temperatures attained at QG are also lower than the ones achieved in Aciprestes, suffering from less aridity and less water deficit in the summer. The predominant grape varieties in this farm are: "Moscatel Galego", "Arinto", "Fernão Pires", "Viosinho" and "Gouveio" (RCV, 2014).

[3] https://whc.unesco.org/en/list/1046/

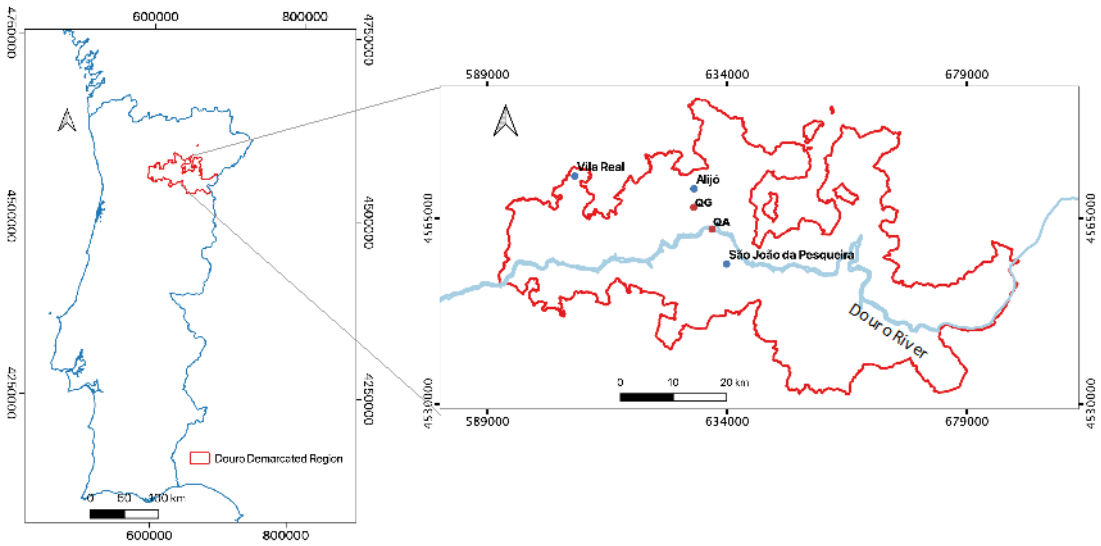


Figure 3.1 The Douro Demarcated Region and location of Quinta dos Aciprestes (QA) and Quinta do Casal da Granja (QG).

Both farms are under integrated production mode (IPM) and, in order to minimize the variables in this work, the choice of each plot was based on the age of the vines (15 years old). Although the same type of phytosanitary treatments is applied in the two vineyards (copper-based fungicides, sulfur, synthetic organic fungicides, and the herbicide glyphosate), the frequency of treatments might be different each year since the two plots differ on the variety of grapes planted: in QA, it was selected a "Touriga Nacional" plot and in QG a “Moscatel Galego” plot. The last one is a very sensitive variety that needs more annual phytosanitary treatments, resulting in two different scenarios of pesticide application. Regarding fertilization, the last application was made in 2016. In both plots inorganic synthetic fertilizers were applied whereas organic fertilization was only made in QG (Annex A1).

3.2.2 Sampling design, samples collection and pre-treatment

Taking into account the purpose of this work, soil samples were collected in four different periods throughout the year of 2018 (Figure 3.2): in February during herbicide application; in April, two months after herbicide application; in June, during fungicide application; and finally, in January 2019, before herbicide application for the next agricultural year. Table 3.1 shows the date and type of treatment performed in each farm during the year of 2018.

Composite soil samples were collected in seven points at QA and in six points at QG (Figure 3.3), for physical-chemical characterization and soil microbial parameters. Within these sampling points, six were selected for pesticide residue analysis and ecotoxicological assays in QA and five in QG. Each composite soil sample consisted in

three sub-samples of superficial soil (0-10 cm): one collected near the stem (in the line) and the other two in each side of the line, about 0.5 meter away.

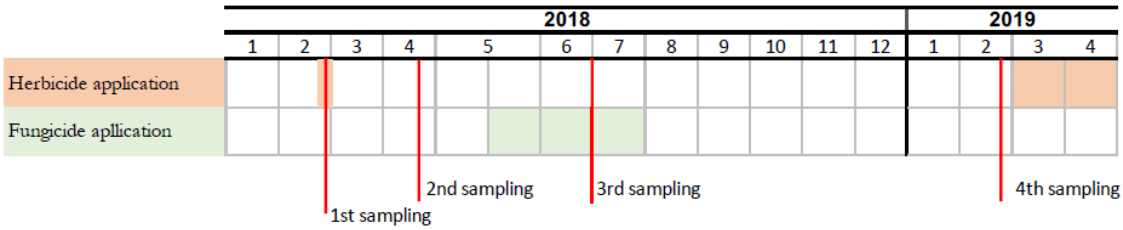


Figure 3.2 Distribution of sampling periods during herbicide and fungicide application for both farms studied.

For the chemical analysis (pH, organic matter, electric conductivity, and inorganic elements), 1 kg of soil was collected and stored in plastic bags until arriving in the lab. Once in the lab, the soil was placed in trays, one per sample, and oven dried for 3 days at 40°C. After this, the samples were manually sieved, and the fraction lower than 2mm was stored at room temperature for analysis. For inorganic elements' analysis, samples were further grounded in an agate mill and then stored in plastic containers, also at room temperature. For these indicators, soil samples were collected only in June 2018 and January 2019.

For PPPs analysis, around 500 g of soil were collected in the field and involved in aluminum foil. Samples were then freeze-dried, sieved to <2 mm, homogenized and frozen (-20 ºC) wrapped again in aluminum foil until analysis. In this case, soil samples from the four sampling periods were analyzed.

For soil microbial parameters samples were stored in plastic bags and refrigerated until arrival to the lab. Once in the lab they were immediately frozen at -20°C. Before the analysis, the samples were slowly thaw at 4°C, manually sieved and the fraction lower than 2 mm was used for analysis. The soil samples collected in February and June 2018 were the only ones analyzed in terms of soil microbial parameters.

For the ecotoxicological assay, around 6 kg of soil was collected to plastic bags and once in the lab, the soil was dried at room temperature until it was possible to manually sieve at <4 mm. Samples were then frozen at -20 °C until testing. This procedure was followed in all soil samples for the four sampling periods.

Table 3.1 Information provided by Real Companhia Velha on the date, plant protection products and corresponding doses applied in both farms studied. Lines in bold represent the sampling period.

Farm	Date	Plant Protection Product (p/p)	Type of treatment	Dose per ha
QA	22-02	Glyphosate 31%	Herbicide	4L
		Flazasulfuron 25%	Herbicide	0.2Kg
	30-04 to 11-05	Folpet 25% + Fosetyl-aluminum 50%	Fungicide	3Kg
		Sulfur 80%	Fungicide	4Kg
	May	Pheromone Diffusers ISONET-L	Sexual confusion	500 dif.
	07-05 to 10-05	Sulfur 98,5%	Fungicide	20Kg
	14-05 to 23-05	Dimethomorph 6% + Folpet 25% + Fosetyl-Al 50%	Fungicide	3Kg
	28-05 to 07-06	Dithianon 35% + Dimethomorph 15%	Fungicide	1.5Kg
		Boscalid 18.2% + Kresoxim-methyl 9.1%	Fungicide	0.3L
	11-06 to 22-06	Copper 14.19% + Metalaxyl-m 2%	Fungicide	5Kg
		Tebuconazole 50% + Trifloxystrobin 25%	Fungicide	0.16Kg
	25-06 to 05-07	**Folpet 25% + Fosetyl-Al 50% + Iprovalicarb 4%**	**Fungicide**	**3Kg**
		Spiroxamine 50%	**Fungicide**	**0.6L**
	09-07 to 20 - 07	Copper oxychloride 40% + Iprovalicarb 8%	Fungicide	1.5Kg
		Kresoxim-methyl 25% + Penconazole 8.75%	Fungicide	0.3Kg
	30-07 to 01-08	Kaolin clay;	Protection against sunburn	25Kg
QG	21-02 to 22-02	Glyphosate 31%	Herbicide	4L
		Flazasulfuron 25%	Herbicide	0.2Kg
	21-05 to 01-06	Dimethomorph 6% + Folpet 25% + Fosetyl-Al 50%	Fungicide	3Kg
		Sulfur 80%	Fungicide	4Kg
	21-05 to 24-05	Pheromone Diffusers ISONET-L	Sexual confusion	500 dif.
	11-06 to 29-06	Sulfur 98.5%	Fungicide	20Kg
	18-06 to 22-06	Spiroxamine 50%	Fungicide	0.6L
	14-05 to 23-05	Dithianon 35% + Dimethomorph 15%	Fungicide	1.5Kg
	25-06 to 06-07	**Dithianon 35% + Dimethomorph 15%**	**Fungicide**	**1.5Kg**
		Boscalid 18.2% + Kresoxim-methyl 9.1%	**Fungicide**	**0.3L**
	09-07 to 20-07	Copper oxychloride 40% + Iprovalicarb 8%	Fungicide	1.5Kg
		Kresoxim-methyl 25% + Penconazole 8.75%	Fungicide	0.3Kg
	23-07 to 30-07	Folpet 25% + Fosetyl-Al 50% + Iprovalicarb 4%	Fungicide	3Kg
		Spiroxamine 50%	Fungicide	0.6L
	09-07 to 20-07	Copper 14.19% + Metalaxyl-m 2%	Fungicide	5Kg
		Tebuconazole 50% + Trifloxystrobin 25%	Fungicide	0.16Kg

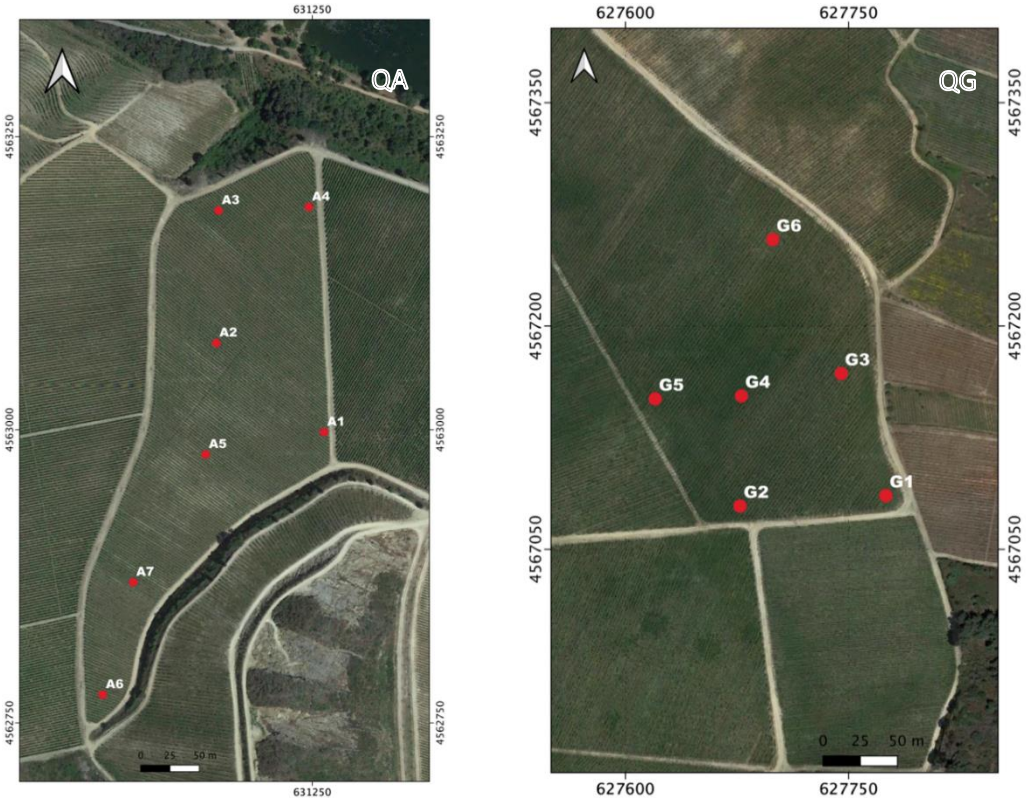


Figure 3.3 Sampling design for both plots studied: Quinta dos Aciprestes (QA) and Quinta da Granja (QG).

3.2.3 Determination of physical-chemical parameters

Soil's pH was measured on soil:water (1:5 m/v) and soil:KCl (1M) (1:5 m/v) suspensions as described in the ISO 10390 standard (ISO, 2002), and electric conductivity was measured in the same soil:water suspension used for pH measurement. Ten grams of soil from each sample were mechanically stirred in 50 ml of deionized water (for pH_w) or KCl solution (for pH_{KCl}), for 5 minutes. This mixture remained resting for about 24 hours and the supernatant's pH was measured using a previously calibrated pH meter (Edge®, Hanna Instruments). The electric conductivity was measured in the same suspension, which was left to rest during the night, using a conductivity meter.

The soil organic matter (OM) was measured by loss-on-ignition, a method that determines total soil organic matter content based on the weight loss of a soil sample, previously dried at 105°C ($Soil_{105°C}$), after ignition at 450°C, for 8 hours ($Soil_{450°C}$). After this period, the crucibles containing the ignited soil samples were left to cool within the furnace, and after that in the desiccator, and then were weighted to the nearest 1mg. The weight of ignited soil was determined. The percentage of organic matter in the soil samples was calculated using equation 1.

$$\text{Total OM\%} = \frac{\text{Soil}_{105^{\circ}\text{C}} - \text{Soil}_{450^{\circ}\text{C}}}{\text{Soil}_{105^{\circ}\text{C}}} * 100$$

(eq 1)

The pseudo-total concentration of major and trace elements was determined by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) (Agilent 7700) after digestion in an heating block (DigiPREP MS, SCP Science), with mixture of HNO_3:HCl (3:1), following the method 3051A from USEPA (Environmental Protection Agency, 2007). The extracts were analyzed for 17 chemical elements: Mg, Al, P, K, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Cd, Ba, and Pb. To evaluate the accuracy and the precision of the analytical method, procedure blanks, and certified reference materials were included in each analytical batch. The replicate analysis of the soil gave an uncertainty of <10% for these inorganic elements. The results of blank analysis were always below the detection limit and recoveries of reference materials (Till 1 and ERM-CC141 LOAM SOIL) were within the certified value.

3.2.4 Determination of Plant Protection Products levels

Glyphosate (Gly) and AMPA determination was performed according to published articles (Bento et al., 2016; Botero-Coy et al., 2013; Yang et al., 2015) with minor modifications. Two grams of soil were weighted into 50 mL centrifuge plastic tubes and 10 mL 0.6 M KOH was added. The tubes were then placed in an end-over-end shaker for 30 min and, at the of that time, soil extracts were centrifuged at 4500 rpm for 10 min. Afterward, 1.5 mL of the supernatant was collected in a new 10 mL centrifuge tube and 1 mL of ultra-pure water and 90 µL of the isotope labelled internal standard $^{13}C,^{15}N$-AMPA and 1,2-$^{13}C_2,^{15}N$- Glyphosate (5 mg L^{-1}) were added. Soil extracts were adjusted to pH 9 by adding HCl 6 M and/or 0.6 M, and the volume was finally made up to 3 mL with ultra-pure water. After this, the extract was loaded onto an OASIS HLB SPE cartridge (200 mg), previously conditioned by passing 2 mL methanol and 2 mL water at pH 9. The non-retained sample extract was collected, and a 2 mL portion was derivatized by adding 120 µL of 1% NH_4OH and 120 µL of 12000 ppm FMOC-Cl. The tubes were shaken for a few seconds in a vortex mixer and incubate for 30 min at room temperature. The reaction was stopped by adding 10 µL of 6M HCl. The tubes were shaken again for a few seconds in a vortex mixer and derivatized extracts were filtered through a 0.45 µm PTFE filters into LC vials. Glyphosate and AMPA were determined by LC-MS/MS using the internal standard method.

The LC-MS/MS system comprised a Waters 2695 XE separation module (Milford, MA) interfaced to a triple quadrupole mass spectrometer (Quattro micro™ API triple

quadrupole, Waters Micromass, Manchester, UK). The LC separation was performed using a core-shell Kinetex C18 column (2.6 µm; 100 x 2.1 mm) at a flow rate of 300 µL min^{-1}. A binary gradient was used, which consisted of solvent A (10 mM acetic acid/ammonium acetate, pH 5.7) and solvent B (methanol). The percentage of organic modifier (B) was changed linearly as follows: 0 – 0.5 min, 10%; 0.5 – 5.5 min, 95%; 5.5 – 8.5 min, 95%; 8.5 – 9 min, 10%; 9 – 14 min, 10%. The injection volume was 20 µL and the column temperature was kept at 40 ºC. The MS parameters were as follows: ion mode, positive; capillary voltage, 3.00 kV; source temperature, 130 ºC; desolvation temperature, 450 ºC; desolvation gas flow, 600 L h^{-1}; and multiplier, 650 V. High purity nitrogen (>99.999%) and argon (>99.999%) were used as the cone and collision gases, respectively. The precursor and product ions as well as the cone voltage and collision energy for each GLY-FMOC, AMPA-FMOC and ILIS-FMOC were determined by flow injection analysis and the MRM transitions, cone voltages and collision energies are listed in Table 3.2. Data acquisition was performed by the MassLynx V4.1 software.

Table 3.2 MRM transitions, cone voltages and collision energies for each GLY-FMOC, AMPA-FMOC and ILIS-FMOC.

Compound	Precursor ion (m z^{-1})	Product ion (m z^{-1})	Cone voltage (V)	Collision energy (V)
GLY-FMOC	392.2	Q:88.0	20	20
		q:170.0	20	10
1,2-^{13}C2, ^{15}N GLY-FMOC	395.2	91.0	20	20
AMPA-FMOC	334.0	Q:112.1	20	15
		q:179.1	20	20
^{13}C,^{15}N-AMPA	336.0	114.1	20	15

Q: quantification transition
q: confirmation transition

QA/QC procedures included the analysis of method blanks, which were always below the detection limit (0.03 mg kg^{-1} for Glyphosate and 0.07 mg kg^{-1} for AMPA), the analysis of spiked samples (recoveries between 91 and 124% for both compounds) and of a soil spiked with a GLY commercial formulation (recoveries between 83 and 107%).

The analysis of other PPPs residues (more than 500 active ingredients) was conducted by an external lab (LAB-SL, Spain). Briefly, the procedure of extraction is based on modified QuEChERS (acetonitrile extraction/partitioning and clean-up by dispersive SPE) and the determination of PPPs residues was performed by GC-MS (7000C Agilent) and/or LC-MS/MS (LC Agilent Model 1290 and MS Sciex® Model 5500).

The calibration was performed on matrix using internal standard, and the identification of PPPs, was based on standard deviation of retention time between the samples and standard (lower than 0.1 min).

3.2.5 Determination of soil microbial parameters

For the determination of the soil's microbial parameters, 1g of fresh soil was weight into 15 mL centrifuge tubes: 3 control and 3 analytical replicates were prepared for each soil sample. Soil moisture content was also determined through the loss of weight after drying at 105°C for 24 hours. The enzymatic activity was measured using the methodologies described by Schinner et al. (1996) and adapted to a microplate reader as previously described by Antunes et al. (2011).

Dehydrogenase activity

For the determination of the activity of dehydrogenases, the samples were suspended in a 1% triphenyltetrazolic chloride solution (prepared in TRIS buffer, 0.1 M) and incubated for 24 hours, at 40°C. The triphenylformazan (TPF) produced was extracted with acetone and measured spectrophotometrically at 546 nm. The control samples were treated in a similar way but instead of adding substrate solution before incubating, TRIS buffer was added. The concentration of TPF produced was determined using a standard calibration curve (absorbance *vs* 10 standard solutions with concentrations ranging from 0 to 100 µg TPF mL^{-1}). The enzyme activity was calculated using equation 2.

$$\text{Dehydrogenases activity} = \frac{\frac{(L - C) \times V}{W} \times \frac{100}{\%dm}}{24} \ (\mu g\ TPF\ .\ g^{-1} dm.\ h^{-1}) \quad \text{(eq. 2)}$$

Where,

L – Mean concentration of the samples (µg TPF mL^{-1})

C – Mean concentration of the controls (µg TPF mL^{-1})

V – Incubation volume (6mL)

W – Initial soil weight (1g)

%dm – Percentage of dry matter (100-%humidity).

Arylsulfatase Activity

For the determination of arylsulfatase activity in soil samples, a 0.02M potassium-p-nitrophenylsulfate solution (prepared with an acetate buffer 0.5M, pH 8.5) was added to the centrifuge tubes which were incubated for 1 hour at 37°C. The nitrophenol (pNP)

released by the arylsulfatase activity was then extracted and colored with sodium hydroxide 0.5M and measured spectrophotometrically at 420nm. The control samples were treated in a similar way, but the substrate solution was added after the incubation. The concentration of p-nitrophenol (pNP) produced was determined using a standard calibration curve (absorbance *vs* 7 standard solutions with concentrations ranging from 0 to 20 µg pNP mL^{-1}). The enzymatic activity was calculated using equation 3.

$$\text{Arylsulfatase activity} = \frac{\frac{(L - C) \times V}{W} \times \frac{100}{\%dm}}{1} (\mu g\ pNP.\ gsoil^{-1}.\ h^{-1})$$

(eq. 3)

Where,

L – Mean concentration of the samples (µg pNP mL^{-1})

C – Mean concentration of the control (µg pNP mL^{-1})

V – Incubation volume (10mL)

W – Initial soil weight (1g)

%dm – Percentage of dry matter (100-%humidity).

Acid phosphatase activity

For the determination of acid phosphatases activity, a buffered solution of p-nitrophenyl phosphate (pH 6.5 with MUB) was added to the samples and then they were incubated for 2h, at 35°C. The p-nitrophenol (pNP) released by phosphomonoesterase activity was extracted with sodium hydroxide, which produces a yellow coloration, measurable spectrophotometrically at 405nm. The control samples were treated in a similar way, but the substrate solution was only added after the incubation period. The concentration of p-nitrophenol (pNP) produced was determined using a standard calibration curve (absorbance *vs* 7 standard solutions with concentrations ranging from 0 to 30 µg pNP mL^{-1}). The enzymatic activity was calculated using equation 4.

$$\text{Acid phosphatase activity} = \frac{\frac{(L - C) \times V \times D}{W} \times \frac{100}{\%dm}}{2} (\mu g\ pNP.\ g^{-1}\ dm.\ h^{-1})$$

(eq. 4)

Where,

L – Mean concentration of the sample (µg pNP mL^{-1})

C – Mean concentration of the control value (µg pNP mL^{-1})

V – Incubation volume (5mL)

D – Dilution factor of the supernatant (2)

W – Initial soil weight (1g)

%dm – Percentage of dry matter (100-%humidity).

Nitrogen mineralization

For the determination of the nitrogen mineralization, the samples were incubated with deionized water for 7 days at 40°C. During this period, the organic forms of nitrogen originate inorganic forms (preponderantly ammonium ion, NH_4^+), which is determined by a modification in Berthelot reaction, after extraction with potassium chloride. This reaction is based in the reaction between sodium salicylate and ammonia (NH_3) in the presence of sodium dichloroisocyanurate, forming a green complex in pH alkaline conditions. The sodium nitroprusside is used as a catalyzer to increase the method's sensibility. The released inorganic nitrogen is measured spectrophotometrically at 690nm. The control samples were treated in a similar way, but they were incubated at -20°C. The concentration of nitrogen (N) produced was determined using a standard calibration curve (absorbance *vs* 6 standard solutions with concentrations ranging from 0 to 1.6 µg NH_4^+ mL^{-1}). The activity was calculated using equation 5.

$$\text{Nitrogen mineralization} = \frac{\frac{(L - C) \times V \times D}{W} \times \frac{100}{\%dm}}{7} \ (\mu g\ N.g^{-1}dm.d^{-1}) \quad \text{(eq. 5)}$$

Where,

L – Mean concentration of the samples (µg N mL^{-1})

C – Mean concentration of the controls (µg N mL^{-1})

V – Incubation volume (6mL)

D – Dilution factor of the supernatant (5)

W – Initial soil weight (1g)

%dm – Percentage of dry matter (100-%humidity).

3.2.6 Ecotoxicological assays

I. Aquatic organisms

A bioluminescence assay with *Aliivibrio fischeri* (Microtox® test) was performed in the context of this work using a Microtox 500 Analyzer, following the protocol provided by

the manufacturer (AZUR Environmental, 1998). The *Basic Solid-Phase Test* was chosen in the software MicrotoxOmni Azur since it is an acute toxicity test, commonly used for solid matrices (soils and sediments). For this assay, the soil suspension was prepared with 17.5 mL of solid-phase diluent and 3.5 g of soil stirred for 10 minutes. After this, 2mL of the soil suspension was placed in a glass cuvette from which a series of dilutions were made. The solid-phase diluent was also placed in glass cuvettes to which the bacteria was added (except in the control) to read the initial bioluminescence (I0) after 15 minutes. After reading I0, the soil suspension dilutions were added to the solid-phase diluent with the bacteria and the bioluminescence was read after 5, 15 and 30 minutes of exposure. With this assay, the EC_{20} and EC_{50} (effective concentrations for 20 and 50% bioluminescence inhibition) were estimated with a 95% confidence interval. However, when it was not possible to estimate ECx values, the effect after 30 minutes of exposure (% of bioluminescence inhibition) at the highest concentration was registered instead.

The *Raphidocelis subcapitata* growth inhibition assay was performed according to, the standard OECD protocol 201 (OECD, 2011). The soil elutriates were made by preparing suspensions of 1:4 (m/v) of the samples with Woods Hole MBL medium: 5 g of soil with 20 mL of MBL medium. The suspensions were left to shake mechanically for 24 hours at room temperature, being centrifuged after at 3900 rpm for 5 minutes. Each elutriate was then tested individually at a concentration of 100%. The microalgae culture was inoculated in MBL medium enriched with vitamins at continuous light exposure and at 24 ± 1°C. After 72 hours of growth, the cells were counted using a Neubauer chamber and the initial algae concentration was adjusted to 1×10^4 cells mL^{-1}. This assay was made in 24-well sterile plates (Figure 3.4) in four replicates plus the control. The first row (A1 to A6) of the plate was filled with 2mL of distilled water and wells 1B, 1C and 1D were used as controls with 900 µL of MBL plus 100 µL of algae. The remaining wells were filled with 900 µL of soil elutriate plus 100 µL of the algae inoculum. The plates were incubated at continuous light and at 24 ± 1°C, for 72 hours, with agitation. After this period, the number of algae in the controls and in the soil elutriates were counted in a Neubauer chamber. The algae growth inhibition was calculated using equation 6.

$$\%\, I_R = \frac{\mu_c - \mu_t}{\mu_c} \times 100 \qquad \text{(eq. 6)}$$

Where,

% Ir – percent inhibition in average specific growth rate;

μ_c – mean value for average specific growth rate (µ) in the control group;

μ_t – average specific growth rate for the treatment replicate.

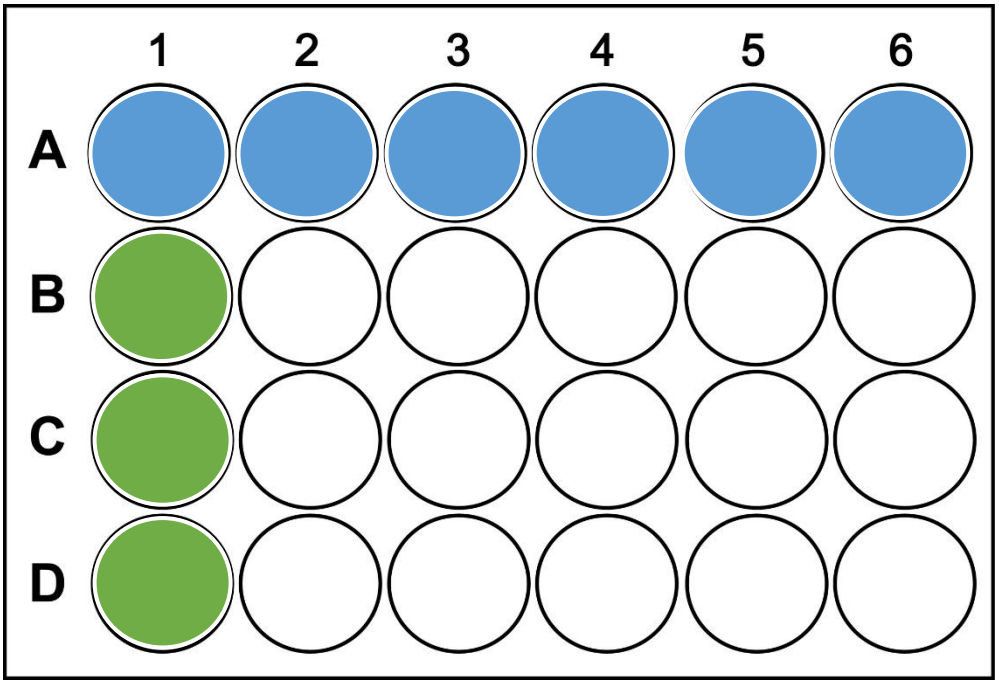


Figure 3.4 Representation of a 24-well plate used in the growth inhibition assay with *Raphidocelis subcapitata*. Blue-wells filled with water; Green- wells of control, White-wells filled with elutriate plus algae.

Lemna minor growth inhibition assay was performed according to the OECD guideline 221 (OECD, 2006b). The aquatic plants for testing were cultured in Steinberg culture medium. Cultures were maintained in an acclimated chamber with controlled photoperiod (16hL:8hD) and temperature (24 ± 2 °C). To perform this assay, soil elutriates were prepared with 12.5 g of the soil sample and 50 mL of Steinberg, stirring for 24 hours. After this period, the elutriate was centrifuged at 3900 rpm for 5 minutes. Each elutriate was then tested individually at a concentration of 100%. A 6-well plate was used in the assays for each sample, three wells were filled with 12 mL of Steinberg (control) and the other three wells were filled with 12 mL of the soil elutriate for that sample. Three plants with three visible fronds were selected and added to each of the six wells and three sets of three plants, from the same culture, were dried at 60°C serving as control of the initial dry weight. After seven days of exposure, the number of fronds were counted and then dried at 60 °C until achieving a stable weight. The growth inhibition rate was calculated according to, the average specific growth rate (equation 7) and yield (equation 8).

$$\%\ I_r = \frac{\mu_c - \mu_t}{\mu_c} \times 100$$

(eq. 7)

Where,

$\%\ I_r$ – percent inhibition in average specific growth rate;

μ_c – mean value for μ in the control;

μ_t – mean value for μ in the treatment group.

$$\%\ I_y = \frac{b_c - b_t}{b_c} \times 100$$

(eq. 8)

Where,

$\%\ I_y$ – percent reduction in yield;

b_c – final biomass minus starting biomass for the control group;

b_t – final biomass minus starting biomass in the treatment group.

II. Terrestrial organisms

For the following ecotoxicological assays, an artificial soil was prepared according to the OECD protocol nº 207 (OECD, 1984) and used as control. This soil was composed of 70% (m/m) sand, 20% (m/m) kaolin and 10% (m/m) peat. The pH_{KCl} of the soil (1:5 m/v) was adjusted to 6.0 ± 0.5 by the addition of calcium carbonate (CaCO3), whenever necessary.

Avoidance assays with the earthworm *Eisenia fetida*, were performed using cultured organisms. The earthworms were maintained in plastic boxes (10–50 L) containing a substrate composed of dry and defaunated (through two freeze–thawing cycles: 48h at -20°C followed by 48h at 65°C) peat, sterilized horse manure, deionized water and $CaCO_3$ to adjust the substrate pH (6.0 ± 0.5). The earthworms were fed every 2 weeks with oatmeal previously hydrated with deionized water. For the avoidance test with *E. fetida*, the ISO 17512-1 (ISO, 2005) was followed as described by Sousa et al. (2008). Rectangular plastic containers were used and divided in two compartments by a removable cardboard split. On the outside of the container a line representing the split place was drawn. The soil used as control, which in this assay was the one collected in January 2019 (outside the application period), was placed in one of the compartments, and the test soil (from February, April, and June 2018) was placed in the other compartment. Soil humidity was adjusted to 50% of the water holding capacity, which was determined as described by Sousa et al. (2008). The artificial soil mentioned in the

beginning of this section was used as for dual control tests in this assay to check the random distribution of organisms when there is no contamination. Thus, in this dual control, avoidance is not expected. Five replicates of each sample and of the dual control were prepared, and 10 organisms, with a weight between 0.30 and 0.60 g, were added to each one. The test containers were kept at 20 ± 2°C and 16h L:8h D photoperiod. After the 48h test period, the split was reintroduced in the marked position and the individuals were counted in each compartment containing the control and the test soil. If any earthworm was not found it was assumed as dead. Earthworms that were cut by the split were considered as being in the soil to which the organism's anterior part was directed to. The percentage of avoidance was calculated according to equation 9 as described in ISO 17512-1 (ISO, 2005). When more than 80% of the test organisms were found in the control soil at the end of the test, the habit function of the soil is considered as being seriously compromised (Hund-Rinke & Wiechering, 2001).

$$\%\ \text{Avoidance} = \frac{\mathrm{C} - \mathrm{T}}{\mathrm{C}} \times 100$$

eq. 9

Where,
C – expected number of worms in control soil;
T – mean number of worms in test soil.

The seedling emergence and seedling growth test with *Medicago sativa* was also performed, following OCED guideline No. 208 (OECD, 2006a). The test was carried out in plastic pots containing 200 g of artificial soil (for control) or of the test soil. Maintenance of soil moisture was ensured by the presence of a pot with distilled water placed at the base of the soil pots with soil, and by using a cotton rope that was in contact with both pots, to ensure the capillarity rise of the water. Twenty seeds of *Medicago sativa* var. Dimitra, acquired from Flora Lusitana Lda (Cantanhede,Portugal) were placed in each pot, after sterilization with 70% (v/v) ethanol (7 min) and 20% (v/v) commercial bleach (5% active chloride; 7 min), followed by washing with deionized water. To ensure the availability of nutrients, a commercial fertilizer (EcoGrow, NPK 3-6-7) was added at the start of the test. Five replicates were prepared for the control and three for the soil samples. The assay began when 50% of the seeds from the control germinated which were counted and registered. In each pot, only ten seedlings were kept, avoiding intraspecific competition. The seeds germinated and grew in a growth chamber with controlled temperature (21°C), photoperiod (16h light/8 h dark) and photosynthetic active

radiation (120 µmol m^{-2} s^{-1}). After 21 days of growth, seedlings from each replicate were collected, and used for the estimation of biometric parameters.

3.2.7 Statistical analyses

The statistical analysis was made using software GraphPad Prism 8. When only two sampling periods were analyzed, namely for the physical and chemical indicators and microbial parameters, an analysis of the variance of two factors (two-way ANOVA) was performed, defining as fixed factors the period and the farm, to test for their significant effect on these indicators. Significant statistical differences were considered when *p* value ≤ 0.05. Not having verified interaction between both factors, an unpaired t-test was performed between sampling periods as well as between farms. Whenever the assumption of variances homoscedasticity was not accomplished, a Mann-Whitney test was performed.

For the levels of plant protection products and the ecotoxicological assays with aquatic organisms, a two-way ANOVA was performed following the same principals as described for the physical and chemical indicators and microbial parameters. Not having verified interaction between both factors, a one-way ANOVA was performed in order to verify if there were significant statistical differences between the four sampling periods. Having verified significant statistical differences between periods, a Tukey's multiple comparisons test was made in order to identify between which periods the significant differences occurred. The Kruskal-Wallis and Dunn's multiple comparisons tests were used when ANOVA assumptions were not met. In order to identify differences between farms for each sampling period, an unpaired t-test or a Mann-Whitney test was performed.

For the avoidance assays, in order to test the hypothesis of no significant avoidance in the dual controls, a two-tailed t-test was performed. Having registered no significant avoidance, a one-tailed Fischer Exact Test was used to test the null hypothesis of no significant avoidance of the test soils.

In the specific case of the plant assays, a one-way ANOVA was performed, followed by a Dunnett's multiple comparison test, in order to verify if there were significant statistical differences between the four sampling periods and the control samples for each farm.

3.3 Results

3.3.1 Physical and Chemical Parameters

Figure 3.5 shows the results obtained for the physical and chemical parameters analyzed for each farm comparing two sampling periods: June 2018 and January 2019. The descriptive statistics of these parameters can be found in Table A2 (Annex). The first indicator analyzed was pH_w and, according to the results presented in Figure 3.5a, it is possible to note that there were significant statistical differences between the two sampling periods for QA ($t = 2.941$, $df = 12$; $p \leq 0.05$). Moreover, the pH_w mean value in QA was slightly higher than the one obtained for QG in both sampling periods, being this difference statistically significant in January 2019 ($t = 2.990$, $df = 11$; $p \leq 0.05$). For pH_{KCL} (Figure 3.5b), no significant statistical differences were observed between sampling periods for both farms. However, the pH_{KCl} mean value in QA was higher when comparing with QG in both periods ($t = 7.361$, $df = 11$ for June; $t = 5.552$, $df = 11$ for January; $p < 0.05$ in both cases).

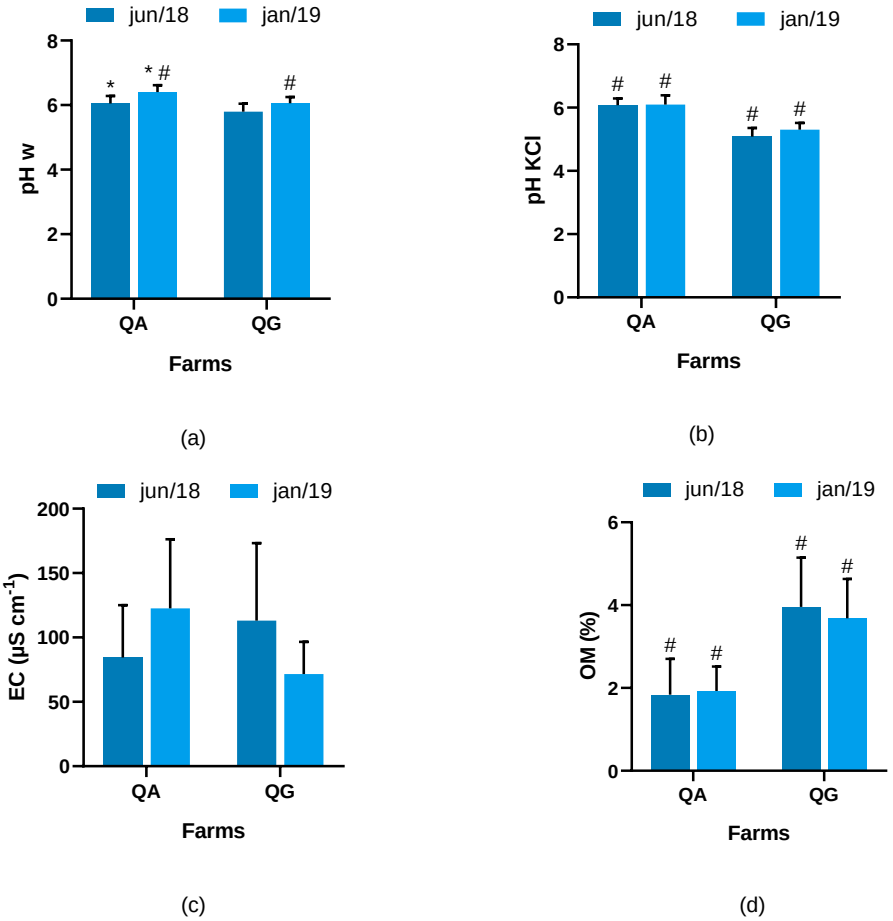


Figure 3.5 Mean values observed for pH_w (a), pH_{KCl} (b), electrical conductivity (c) and organic matter (d) in the two farms studied, for the two sampling periods. Error bars correspond to the standard deviation. Statistically significant differences between sampling periods for the same farm, considering $p \leq 0.05$, are marked with * above bars. Statistically significant differences between farms for the same sampling period, considering $p \leq 0.05$, are marked with # above bars.

Regarding the electric conductivity (Figure 3.5c), the results obtained were different in both farms: in QA there was an increase between June 2018 and January 2019 and the opposite happened in QG. Despite this, no significant statistical differences were observed in both farms for the two sampling periods.

Another parameter measured was the organic matter content (Figure 3.5d) which showed no significant statistical differences in both farms between sampling periods. Still, the organic matter mean value was higher in QG comparing to QA ($t = 3.715$, $df = 11$ for June; $t = 4.081$, $df = 11$ for January; $p \leq 0.05$ in both cases).

Figure 3.6 shows the concentrations of macronutrients (Mg, P, K and Ca) and micronutrients (Mn, Cu, Zn and Ni) evaluated and in Table A3 and A5 (Annex) the descriptive statistics of all elements analyzed.

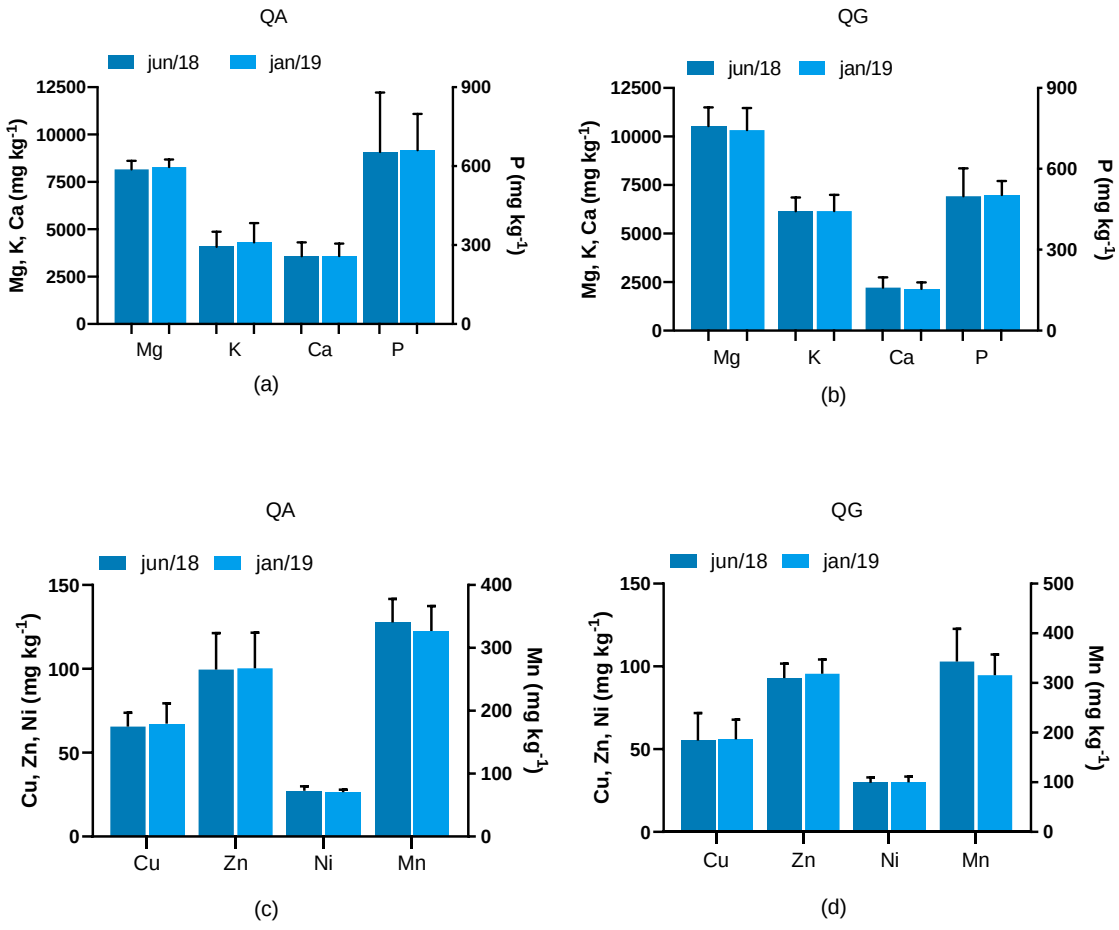


Figure 3.6 Mean values of total content of macro (a and b) and micronutrients (c and d), for two sampling periods in both farms studied. Error bars correspond to the standard deviation.

No significant statistical differences between sampling periods were obtained for any of the macronutrients assessed, however, it is possible to note that, despite the high variability in the results obtained, QA had higher average P and Ca levels while QG had

higher average Mg and K content ($p \leq 0.05$, Table A4). Regarding the micronutrients, no significant statistical differences were observed between periods for both farms. However, as can be seen in Table A6, significant statistical differences were recorded between farms for some of the trace elements analyzed (Ni, V, Cr, Ba, Pb).

3.3.2 Levels of Plant Protection Products

The results concerning the quantification of detected PPPs are presented in Table 3.3 (for QA) and Table 3.4 (for QG). From the more than 500 PPPs analyzed (Table A7 and A8, Annex), only 17 were detected. The herbicides detected were Glyphosate, Oxyfluorfen and 2,4-D, whereas the fungicides detected were Boscalid, Dimethomorph, Fluopicolide, Fluopyram, Iprovalicarb, Metalaxyl, Metrafenone, Penconazole, Phthalimide, Spiroxamine, Tebuconazole. Indoxacarb, Kresoxim-methyl and Pyrethrin were the insecticides detected. Regarding the number of detected PPPs (Figure 3.7), in QA this number was higher in February and June 2018, contrary to QG where the highest number was detected in February and April 2018. It should be noted that, with exception of June 2018, the number of detected pesticides was higher in QG comparing to QA.

Regarding the levels of herbicides found, the concentrations of glyphosate and its degradation product AMPA (Table A9) and oxyfluorfen are shown in Figure 3.8. It is possible to note that February 2018 had the highest concentration of GLY in both farms (Kruskal-Wallis = 7.918 $p \leq 0.05$), decreasing significantly only in January 2019 for QA, whereas for QG this value decreases from June. In addition, comparing QA and QG, despite the higher mean value observed in February in the former, no statistically significant differences were observed between farms. Regarding AMPA concentrations (Figure 3.8b), no significant statistical differences were observed between sampling periods in both farms. Despite the higher concentrations observed in QG comparing to the ones obtained in QA in all sampling periods, significant differences were observed in February (Mann-Whitney= 5, $p \leq 0.05$) and April (Mann-Whitney = 3, $p \leq 0.05$). As for oxyfluorfen levels (Figure 3.8c), these follow a tendency similar to GLY: the highest concentration was obtained in February 2018 for both farms, decreasing over time, being the differences statistically significant especially between February and June (Kruskal-Wallis statistic of 15.01 for QA and 14.57 for QG; $p \leq 0.05$). Furthermore, the concentrations registered in QA were slightly higher than the ones registered in QG for all sampling periods, but these differences were not statistically significant.

The total concentration of fungicides in QA and QG are shown in Figure 3.8d and the descriptive statistics can be found in Table A10 (Annex). The concentration follows a similar pattern in both farms: the highest concentration was recorded in June 2018 when

fungicides were applied and the lowest in April 2018, being these differences statistically significant only in QA ($F(3, 20) = 6.838$; $p \leq 0.05$).

Table 3.3 List of the mean and standard deviation (STD) of PPPs detected as well as the number of samples (N in a total of 7 for glyphosate and 6 for the remaining) in which PPPs were detected in QA in the four sampling periods. Names in bold correspond to the PPPs applied in this farm.

		QA							
	PPP	Feb/18		Apr/18		Jun/18		Jan/19	
		Mean±STD	N	Mean±STD	N	Mean±STD	N	Mean±STD	N
Herbicides (mg kg^{-1})	**Glyphosate**	2.78±3.03	7	0.56±0.42	6	0.67±0.84	6	0.14±0.10	7
	Oxyfluorfen	1.23±1.02	6	0.628±0.349	6	0.188±0.138	6	0.087±0.066	5
	2,4-D	0.008±0.002	5	-	-	0.008	1	-	-
Fungicides (mg kg^{-1})	**Boscalid**	0.011±0.006	5	0.012±0.007	4	0.031±0.010	6	0.040±0.027	6
	Dimethomorph	0.163±0.059	6	0.102±0.617	6	0.352±0.178	6	0.172±0.078	6
	Fluopicolide	0.100±0.040	6	0.058±0.02	6	0.038±0.010	6	0.023±0.007	6
	Fluopyram	0.009±0.001	4	0.006±0.001	3	0.007±0.000	2	0.005	1
	Iprovalicarb	-	-	0.012	1	0.040±0.038	6	-	-
	Metalaxyl	-	-	-	-	-	-	-	-
	Metrafenone	-	-	-	-	-	-	-	-
	Penconazole	0.011±0.004	6	0.007±0.001	3	0.011±0.004	4	0.008±0.003	4
	Phthalimide	-	-	-	-	0.016±0.001	2	-	-
	Spiroxamine	0.018±0.060	6	0.024±0.020	6	0.076±0.04	6	0.020±0.010	6
	Tebuconazole	0.006±0.001	2	-	-	-	-	0.007±0.002	3
Insecticides (mg kg^{-1})	Indoxacarb	-	-	-	-	-	-	-	-
	Kresoxim-methyl	-	-	-	-	0.008	1	-	-
	Pyrethrin	0.015	1	0.013	1	0.053	1	0.022±0.023	2

Table 3.4 List of the mean and standard deviation (STD) of PPPs detected as well as the number of samples (N in a total of 6 for glyphosate and 5 for the remaining) in which PPPs were detected in QG in the four sampling periods. Names in bold correspond to the PPPs applied in this farm.

		QG							
	PPP	Feb/18		Apr/18		Jun/18		Jan/19	
		Mean±STD	N	Mean±STD	N	Mean±STD	N	Mean±STD	N
Herbicides (mg kg^{-1})	**Glyphosate**	1,47±0,56	6	0,75±0,45	6	0,39±0,46	6	0,16±0,12	6
	Oxyfluorfen	0.484±0.219	5	0.39±0.157	5	0.162±0.058	5	0.049±0.021	4
	2,4-D	-	-	-	-	-	-	-	-
Fungicides (mg kg^{-1})	**Boscalid**	0.0222±0.017	4	0.0173±0.013	4	0.0143±0.007	3	0.0193±0.014	3
	Dimethomorph	0.188±0.118	5	0.131±0.0577	5	0.180±0.0632	5	0.149±0.0796	5
	Fluopicolide	0.008	1	0.006±0.001	2	-	-	-	-
	Fluopyram	0.0146±0.006	5	0.0134±0.006	5	0.009±0.002	4	0.008±0.004	4
	Iprovalicarb	-	-	-	-	-	-	-	-
	Metalaxyl	0.0318±0.012	5	0.0678±0.005	5	0.149±0.163	5	0.016±0.003	5
	Metrafenone	0.013±0.004	4	0.008±0.003	5	0.007±0.001	2	0.007	1
	Penconazole	0.0106±0.002	5	0.009±0.002	5	0.007±0.001	2	0.007±0.003	4
	Phthalimide	-	-	-	-	0.009	1	-	-
	Spiroxamine	0.016±0.077	5	0.0132±0.005	5	0.117±0.081	5	0.0304±0.012	5
	Tebuconazole	0.0108±0.004	5	0.009±0.002	3	0.007±0.003	3	0.007±0.002	5
Insecticides (mg kg^{-1})	Indoxacarb	0.0152±0.008	5	0.016±0.003	4	0.0095±0.004	2	0.011±0.003	4
	Kresoxim-methyl	-	-	-	-	-	-	-	-
	Pyrethrin	-	-	-	-	-	-	-	-

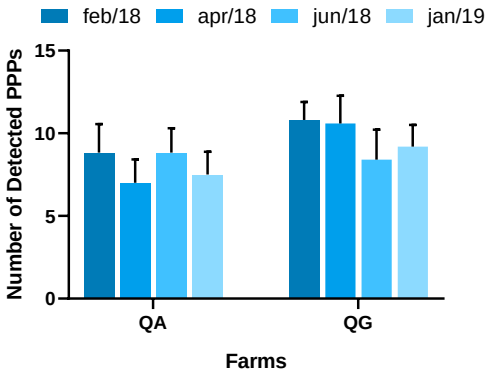


Figure 3.7 Mean number of detected plant protection products in the samples collected in the four sampling periods for both farms studied. Error bars correspond to the standard deviation.

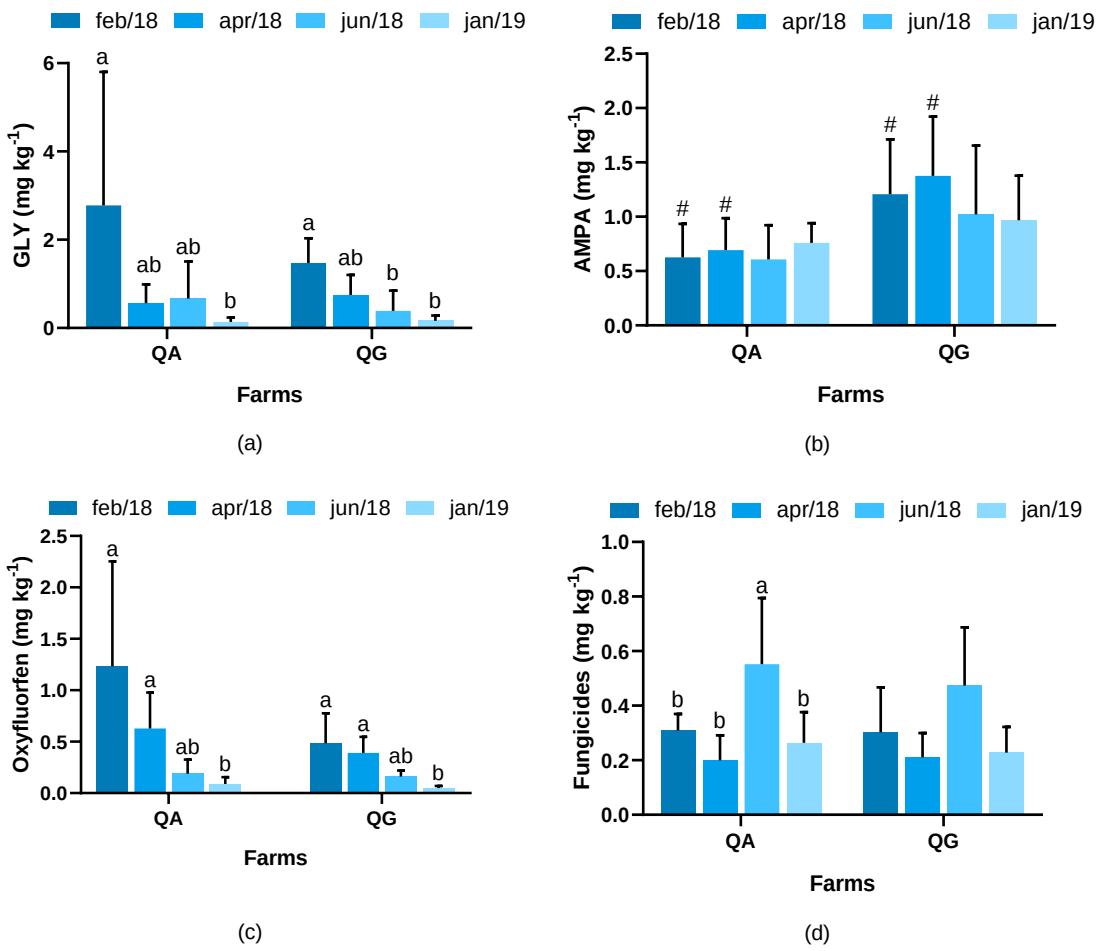


Figure 3.8 Average concentrations of glyphosate (a), AMPA (b), oxyfluorfen (c) and detected fungicides in the four sampling periods for both farms. Error bars correspond to the standard deviation. Statistically significant differences between sampling periods for the same farm, considering p ≤ 0.05, are marked with different letters above bars. Statistically significant differences between farms for the same sampling period, considering p ≤ 0.05, are marked with # above bars.

3.3.3 Microbial Parameters

The results obtained for dehydrogenase, arylsulfatase, and acid phosphatase activity and nitrogen mineralization in February and June 2018 are presented in Figure 3.9. For dehydrogenase activity (Figure 3.9a) in QA, no significant statistical differences were observed between sampling periods, despite the apparent increase of this enzyme's activity between February 2018 and June 2018. The same pattern was registered for QG being the difference, in this case, statistically significant (Mann-Whitney = 0; $p \leq 0.05$). Moreover, in spite of the higher mean value of dehydrogenase activity observed for QG in June 2018 when comparing with QA, the differences were not significant. The arylsulfatase activity (Figure 3.9b) presented similar values in both farms with an activity significantly higher in February comparing to June (t = 3.882, df = 12 for QA; t = 4.368, df = 10 for QG; $p \leq 0.05$). In the case of acid phosphatase activity (Figure 3.9c), significant

statistical differences were only observed for QG (t = 4.504, df = 10; $p \leq 0.05$), being this activity higher in June 2018. In QA, even though no significant statistical differences were registered, the activity was also higher in June comparatively to February 2018. Considering the differences between farms, these were only observed in June (t = 4.423, df =1 1; $p \leq 0.05$).

Regarding nitrogen mineralization (Figure 3.9d), no significant statistical differences were observed for each farm between the two sampling periods, neither between farms. Nevertheless, for QA there was a decrease in mineralization between February and June 2018 whereas in QG there was a slightly increase in the same period.

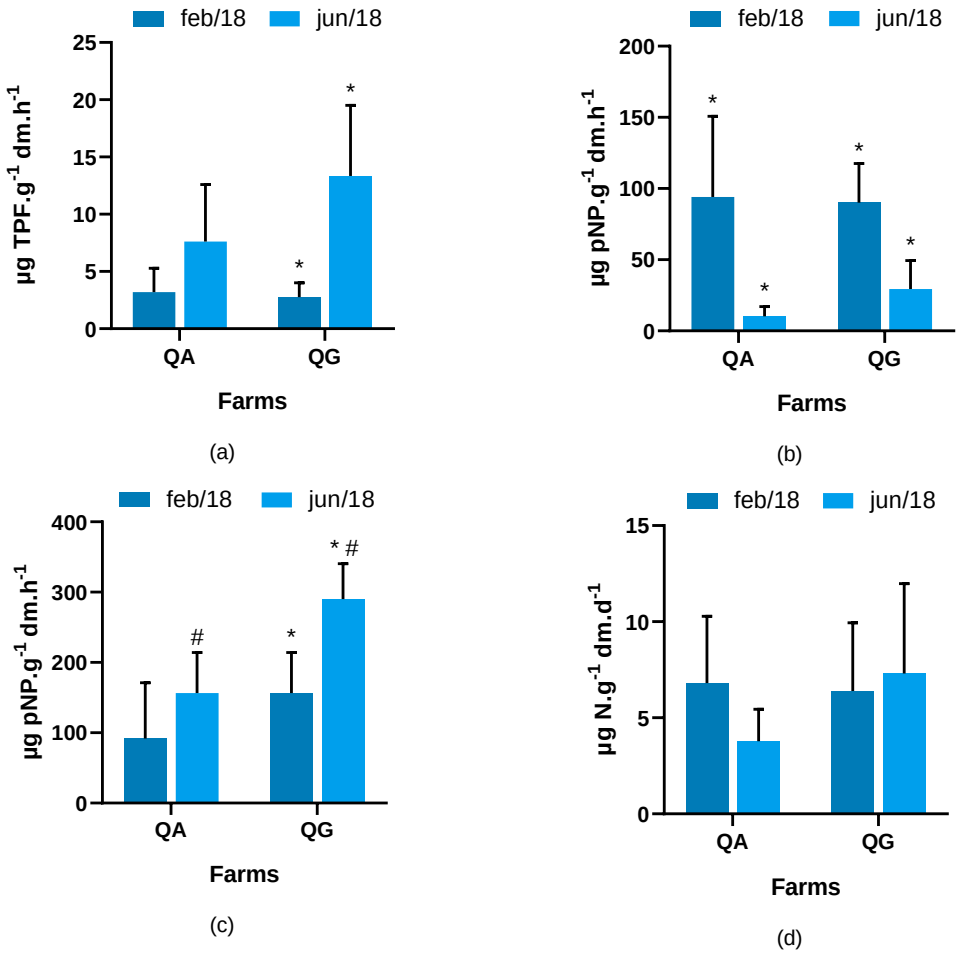


Figure 3.9 Average dehydrogenase (a), arylsulfatase (b) and acid phosphatase (c) activities and average nitrogen mineralization (d) in both farms studied, in two different periods. Error bars correspond to the standard deviation. Statistically significant differences between sampling periods for the same farm, considering p ≤ 0.05, are marked with * above bars. Statistically significant differences between farms for the same sampling period, considering p ≤ 0.05, are marked with # above bars.

3.3.4 Ecotoxicological assays with aquatic organisms

The results obtained for the bioluminescence assay with *Aliivibrio fischeri* (Microtox® test) are presented in Table 3.5. In Table 3.6, the results obtained for the concentration of herbicides and fungicides as well as for the Microtox® test are presented for each sample of both farms which will allow us to discuss these results further ahead. The EC_x values were not determined because percentages of inhibition of 20% or 50% were only recorded at the highest concentrations. In QG there was an increase in the mean effect percentage from February 2018 and January 2019. In QA there was also an increase in the mean effect percentage from February 2018 to June 2018, later decreasing in January 2019. Still, the mean percentage of the effect was higher in QG farm than QA farm.

For the *Raphidocelis subcapitata* growth inhibition assay, the results can be observed in Figure 3.10. The behavior was similar in both farms: the highest inhibition was obtained in February 2018, when herbicides were applied, followed by a decrease over time. Moreover, the growth inhibition was more pronounced in QA farm, where differences between sampling periods were statistically significant ($F(3, 19) = 6.676$; $p \leq 0.05$), comparing to QG. No significant statistical differences were recorded between farms for this ecotoxicological assay.

Table 3.5 Descriptive statistics of percentage of effect obtained in the Microtox® test for the four sampling periods in both farms studied.

Farm	Sampling period	Mean	STD	CV (%)	Median	Min	Max
QA	Feb/18	3.50	18.52	529	0.635	-21.17	27.46
	Apr/18	6.31	23.29	369	6.39	-29.97	31.45
	Jun/18	10.86	15.09	139	7.69	-3.83	33.13
	Jan/19	9.82	15.26	156	7.55	-6.87	25.58
QG	Feb/18	10.42	26.40	253	10.04	-17.73	52.39
	Apr/18	16.94	23.19	137	8.08	-3.61	56.35
	Jun/18	22.38	26.37	118	27.46	-10.30	59.63
	Jan/19	30.33	10.72	35.3	32.97	16.99	42.47

Table 3.6 Results obtained for herbicide (Herb) and fungicide (Fung) concentration and bioluminescence assay with *Allivibrio fischeri* (Mic) for each sample of the two farms studied and the four sampling periods.

Sample	Feb/18			Apr/18			Jun/18			Jan/19		
	Herb (mg kg^{-1})	Fung (mg kg^{-1})	Mic (%)	Herb (mg kg^{-1})	Fung (mg kg^{-1})	Mic (%)	Herb (mg kg^{-1})	Fung (mg kg^{-1})	Mic (%)	Herb (mg kg^{-1})	Fung (mg kg^{-1})	Mic (%)
QA1	0.53	0.044	-3.83	0.81	0.128	14.57	0.10	0.05	10.6	0.25	0.166	-6.87
QA4	0.87	0.050	5.1	1.3	0.188	-1.79	0.20	0.06	-3.7	0	0.137	7.55
QA11	10.01	0.047	-8.35	0.75	0.203	28.70	2.19	0.07	33.1	0.25	0.334	25.58
QA12	3.46	0.100	-21.17	2.01	0.119	-5.08	0.32	0.12	-3.8	0.343	0.313	-2.64
QA16	0.47	0.043	21.8	0.49	0.372	31.45	1.47	0.12	24.2	0.035	0.206	25.46
QA17	5.65	0.050	27.46	1.79	0.188	-29.97	0.72	0.09	4.8	0.40	0.428	-
QG1	1.63	0.021	12.18	1.59	0.207	16.75	0.33	0.11	6.3	0.12	0.378	16.99
QG4	2.42	0.055	52.39	1.58	0.354	7.11	0.76	0.06	59.6	0.406	0.244	42.47
QG6	2.35	0.026	-4.79	1.60	0.15	8.08	1.39	0.13	27.5	0.325	0.201	21.68
QG10	1.08	0.057	10.04	0.52	0.218	56.35	0.29	0.04	-10.3	0.137	0.19	32.97
QG12	2.88	0.028	-17.73	0.98	0.122	-3.61	0.30	0.07	28.8	0.167	0.125	37.56

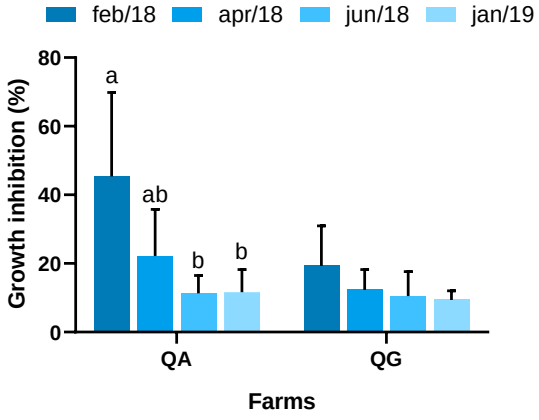
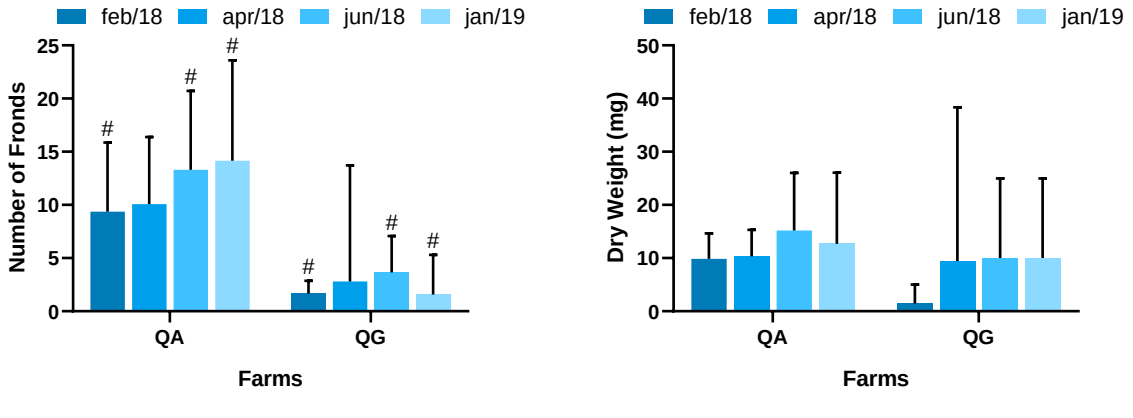


Figure 3.10 Average percentage of *R. subcapitata* growth inhibition for all sampling periods in both farms studied. Error bars correspond to the standard deviation. Statistically significant differences between sampling periods, considering $p \leq 0.05$, are marked with different letters above bars.

Figure 3.11 shows the results obtained for *Lemna minor* growth inhibition assay in terms of number of fronds (a) and dry weight (b). The results showed, for the number of fronds, an increase in inhibition from February 2018 to January 2019 in QA farm. As for QG, the results obtained showed an increase in the inhibition from February to June 2018 followed by a decrease in January 2019. Moreover, significant statistical differences were registered between farms ($t = 2.593$, $df = 9$ for February; $t = 2.669$, $df = 9$ for June and $t = 2.770$, $df = 8$ for January; $p \leq 0.05$ in all cases). Regarding the dry

weight, in QG there was an increase in inhibition between February and April 2018, however, in QA the most notorious increase was between April and June 2018. Nevertheless, no significant statistical differences were registered between sampling periods in each farm. Still, the inhibition for both parameters was slightly higher in QA farm comparing to QG even though no significant statistical differences were observed between farms regarding the dry weight.



(a) (b)

Figure 3.11 Average number of fronds (a) and dry weight inhibition (b) and of *L. minor* during a one-year monitoring period in both farms studied. Error bars correspond to the standard deviation. Statistically significant differences between farms for the same sampling period, considering $p \leq 0.05$, are marked with # above bars.

3.3.5 Ecotoxicological assays with terrestrial organisms

For the avoidance assay with *Eisenia fetida*, the results are shown in the Figure 3.12. In QA there was an increase in the percentage of avoidance over time. As for QG farm, February 2018 was the only sampling period that registered an avoidance response of oligochaetes, reaching 45%. As for April and June 2018 in QG there was no avoidance, in fact, there was a higher number of earthworms found in the test soil, when compared with the soil from January used as CTL. No significant statistical differences were found between sampling periods (excluding January 2019 used as CTL in this assay) for both farms.

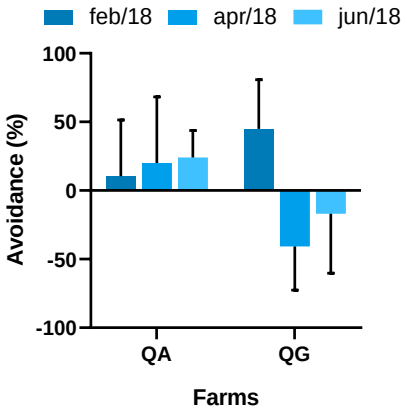


Figure 3.12 Results obtained for the average percentage of avoidance in the avoidance assay with *Eisenia fetida* for both farms in the four sampling periods. Error bars correspond to the standard deviation.

In Figure 3.13 are presented the results obtained for the percentage of germination (a) and survival (b) for the seedling emergence and seedling growth test with *Medicago sativa*. It is possible to note that the germination of seeds added to QA soils were more affected than the ones added in QG soils, having significant statistical differences in February 2018 comparing to the control only in QG. In terms of number of plants that survived during the assay, once again, the plants thar grew in QA soil proved to be more susceptible reaching the lowest percentage of survival in February 2018.

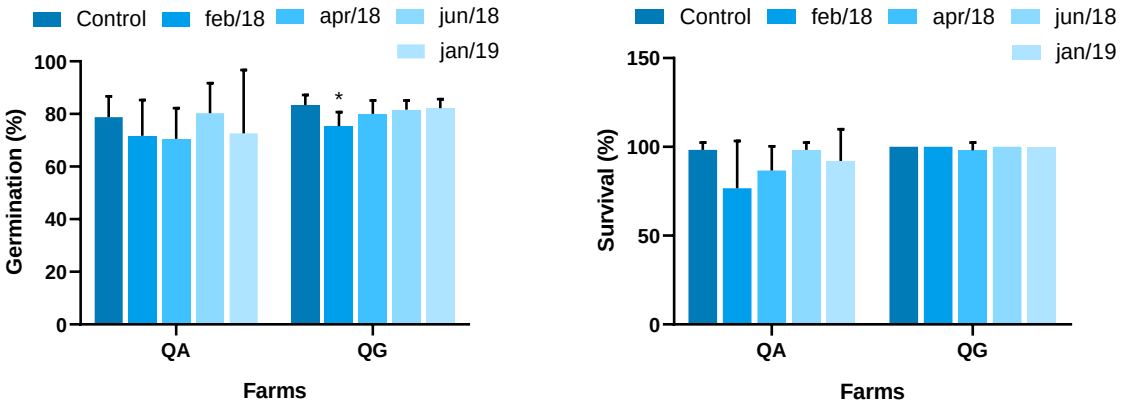


Figure 3.13 Average percentage of germination (a) and survival (b) of *M. sativa* plants in the control and the four sampling periods for both farms studied. Error bars correspond to the standard deviation. Statistically significant differences between the control and the sampling periods for each farm tested individually, considering $p \leq 0.05$, are marked with * above bars.

Regarding the biometric parameters, these are presented in Figure 3.14. Root length (a) and root fresh biomass (b) showed the same response in both farms, however in QA significant statistical differences ($F(4, 24) = 5.036$; $p \leq 0.05$) were obtained in February

and June 2018 for root length and between February, April, and June 2018 for root fresh biomass (F (4, 20) = 1.856; $p \leq 0.05$) whereas in QG no significant statistical differences were recorded between sampling periods.

For shoot growth, once again the response was similar in length (c) and fresh biomass (d) in both farms: significant statistical differences were obtained in February and April 2018 comparing with the control in QA whereas no significant statistical differences were observed in QG.

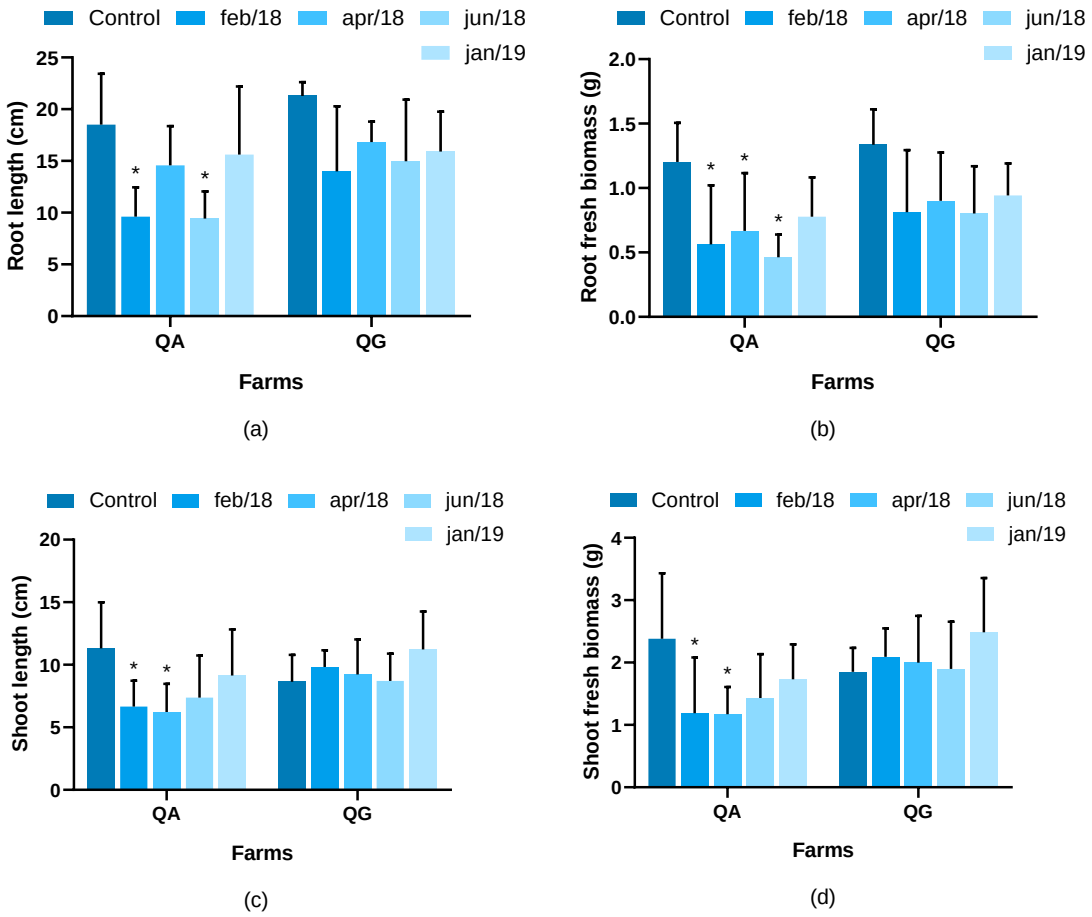


Figure 3.14 Average root length (a) and fresh biomass (b) and shoot length (c) and fresh biomass (d) of *M. sativa* plants for the control and the sampling periods in both farms studied. Error bars correspond to the standard deviation. Statistically significant differences between the control and the sampling periods, considering p ≤ 0.05, are marked with * above bars.

3.4 Discussion

3.4.1 Physical and Chemical Parameters

To this point, there is no information about the levels of PPPs in soils and on how its application affects soils quality in the DDR. In order to better understand the behavior of these contaminants in soils and evaluate their impacts on physical and chemical

properties, the pH, electric conductivity (EC), and organic matter (OM) content were measured. Soil pH is a relevant chemical parameter due to its influence in a range of soil chemical processes: it affects the bioavailability of inorganic elements as well as soil organisms and their activity (Riches et al., 2013). Soil fertility is also affected by this chemical indicator due to its effect in the solubility of cations such Al, Mn, Fe, Cu, Zn and Mo, interfering with their supply to plants. If soil's pH is acid, it may lead to nutrient deficiencies of the major elements such as Ca, Mg, K and P, on the other hand the solubility, and subsequently the bioavailability of potentially toxic elements tends to be higher (Riches et al., 2013). The pH values below 4.0 and higher than 9.0 inhibit root growth (White, 2015) so, the suggested optimum pH_w range for vine growth is between 5.5 and 8.0 (White, 2003). The values measured for both farms and sampling periods in this study were included within this range. According to, the results obtained for pH_w for QA in June 2018 and January 2019 (Table A2), this soil can be classified as slightly acidic, whereas QG soils were classified as moderately acidic (Webster, 2007). The lower pH value observed in these farms in June 2018 comparing with January, especially in QA, (Figure 3.5a) might be related to fungicide application in that period, especially sulfur, as also observed by other authors (Hinckley et al., 2011; Slaton et al., 2001).

The pH was also measured in a KCl suspension (potential acidity) which is based on the release of cations from the soil colloids, such as protons that are replaced by K^+ ions on the soil particles. This process forces protons to move into the soil solution providing a measure of the potential acidity of soil. Thus, the pH values measured in a KCl solution are in average one pH unit lower than the ones obtained from a water suspension (Gavriloaiei, 2012; Hamza, 2008). Contrary to what was observed for pH_w, the pH_{KCl} remained similar between the two sampling periods (Figure 3.5b), but the differences between the two farms are more evident, which may reflect the difference of some other soil properties such as OM, as further discussed.

The EC gives an indication of the salinity of the soil, expressing the concentration of dissolved salts in a given volume of soil. Mineral ions such as Ca^{2+}, Mg^{2+}, Na^+, Cl^-, SO_4^{2-} and HCO_3^- are the ones that most contribute to soil salinity, as well as K^+ and NO^{3-} but in less extent (White, 2003). In order to relate the effect of salinity in crops under field conditions, the soil suspension salinity ($EC_{1:5}$) must be converted to saturation salinity (EC_e) (Hamza, 2008). Since these soils are classified as sandy loams (Costa, 2018), the multiplier factor is 14. Thus, the soils of both farms are classified as non-saline as EC_e values were lower than 2. The results obtained in this study showed a very high variability and an inconsistency: there was an increasing trend in EC in QA and a decreasing pattern in QG between June and January (Figure 3.5c). Since fungicides are applied in

June 2018, it was expected a higher EC in that period, as also reported by Slaton et al. (2001), however, that was only observed in QG.

Organic matter (OM) plays a vital role in biological, chemical, and physical soil properties (Hamza, 2008). It comprises all the living, dead and decomposing plants, animals, and microbes along with the organic residues and humic substances they release. OM plays an important role in the soil structure and porosity; the water infiltration rate and water holding capacity of soils; the diversity and biological activity of soil organisms and plant nutrient availability (Bot & Benites, 2005). The OM content of agricultural top soils should be between 1 to 6% (Magdoff & Es, 2000), however, in the specific case of viticulture, the ideal OM content of soils should be between 2 to 4% (Edwards et al., 2013; Kurtural, 2007). According to Figure 3.5d and Table A2 the OM content in QG reached mean values closed to 4% whereas QA did not reach 2%. However, in a previous study conducted by Costa (2018), in these same farms, in January 2017 the OM content recorded was much higher (4.78 ± 1.59 for QA and 6.75 ± 1.36 for QG) than the ones obtained in this study for the same sampling period (1.93 ± 0.59 for QA and 3.68 ± 0.95 for QG).However, differences observed between the two farms remain similar, i.e., significantly higher values in QG. According to what was communicated by RCV, the last fertilization was made in 2016, but only in QG an organic fertilizer with 50% of organic matter was applied (Table A1). Moreover, the slope of both farms can also explain the results obtained for OM content. As previously reported by Rodrigo Comino et al. (2016) and Biddoccu et al. (2018), the higher the slope, the higher the soil erosion which contributes to the decrease of OM content (Frye et al., 1982). Since QA is located in a slope of 30-50% and in QG the slope is about 15%, it would be expected the OM content to be lower in QA as the slope is higher in this farm, which is in agreement with the results obtained in this study.

Soil fertility is directly related with the content of macronutrients and micronutrients presented in the soil. Even though these nutrients are very important to plant growing and quality of its products, some of these, such as Cu and Zn, are considered potentially toxic elements, and they can be released from chemical fertilizers and PPPs application, contaminating the soil (Milićević et al., 2017; Tiecher et al., 2016). According to Figure 3.6, no significant statistical differences were observed between sampling periods for the macronutrients, however, there was significant differences between farms. Although no fertilization was made during the sampling period, in QG farm, organic and inorganic fertilizers were applied in 2016 whereas in QA only inorganic fertilizer was applied. This could explain the differences obtained between farms for the macronutrients. Furthermore, even though these soils are geologically similar (Costa, 2018), the differences observed for Al and Fe (typically geogenic elements) presented in Table A3,

may indicate that the geology is different between the two locations. Regarding the micronutrient's levels, as observed for the macronutrients, there were no changes between sampling periods but some of them showed significant differences between farms. Once again, these differences could be related to the application of fertilizers or to the farm's geology. In the specific case of Cu, Zn and Mn levels found in these soils, the results obtained could be related to the application of fungicides namely mancozeb and copper-based. It is important to note that, according to Ministério Regional do Ambiente do Ordenamento do Território e do Desenvolvimento (2009), the maximum values of the potentially toxic elements obtained (Cu 83.46 mg kg^{-1}; Ni 35.36 mg kg^{-1}; Zn 145.1 mg kg^{-1}) were below the limit values allowed on soils for agriculture (for pH between 5.5 and 7, Cu 100 mg kg^{-1}; Ni 75 mg kg^{-1}; Zn 300 mg kg^{-1}), thus, they are expected to pose no risks to the environment, at least individually.

3.4.2 Levels of Plant Protection Products

The evaluation of PPPs concentration in the soils over one year (four different sampling periods), allowed to contribute for the understanding about the behavior of these contaminants. During 2018, 8 treatments with PPPs, corresponding to 17 different active ingredients, were performed in QA; whereas in QG the number of treatments was 9, but the number of active ingredients was the same. Despite this, the number of detected PPPs was in general higher in QG (Figure 3.7). Moreover, as can be seen in Table 3.3 and Table 3.4, not every PPP that was applied was detected and other products were detected without being applied. In QA, the highest number was recorded in February and June 2018 and February and April 2018 for QG. It was not expected that February 2018 had the highest number of PPPs since only GLY and flazasulfuran were applied. This could be the result of the number of PPPs applied in the previous year as well as the weather conditions since with less rain there will be less leaching and therefore accumulation in soils. As for June 2018, it would be expected a greater difference in the number of PPPs since more were applied in that period. However, that was not observed which could indicate that, for the most part, the PPPs applied were not retained in the soils.

Glyphosate (GLY) together with flazasulfuran were the only herbicides applied in both farms, however the last one was not detected in any of the soil samples analyzed. GLY is metabolized by microorganisms to AMPA (secondary metabolite) which is then degraded/mineralized to CO_2 (Roberts et al., 1998). Despite its strong adsorption to the soil, GLY degrades at a high rate and has a relatively short average half-life of 30 days that can range between 2 to 215 days (Battaglin et al., 2014; Grunewald et al., 2001b; Maqueda et al., 2017; Monsanto, 2014). This is supported by the results obtained in this

work since the highest GLY concentration was registered in February 2018 for both farms (Figure 3.8a), this being the period of herbicide application. After this period, there was a decrease in concentration over time, reaching less than 0.2 mg kg^{-1} in January 2019. In Europe, Gly-based herbicides are usually applied one to three times a year in vineyards at recommended rates between 0.72 and 2.88 kg ha^{-1} per treatment, and at a maximum annual application rate of 4.32 kg ha^{-1} (EFSA, 2015, 2017). In this study, a single annual application of 1.24 kg ha^{-1} was made, being within the recommended rate. In a study conducted by Silva et al. (2018) regarding the distribution of GLY and AMPA in agricultural topsoil of the European Union, both GLY and AMPA had a maximum concentration of 2 mg kg^{-1} being the soils of Portuguese vineyards the ones with the highest concentration of this herbicide. These values are in accordance with the ones of the present study, despite the slightly higher mean values found in QA samples during February 2018 (2.78 ± 3.03 mg kg^{-1}). Indeed, soil samples from this vineyard, showed a high variability of results, reaching a maximum concentration of 8.50 mg kg^{-1} (Table A9). Regarding the metabolite AMPA, its concentration did not vary significantly between sampling periods (Figure 3.8b) indicating that only a fraction of the applied GLY was retained in the soil and transformed into AMPA. The significant statistical differences between farms observed for the AMPA concentration could be due to the OM content since, as QG had higher OM content, more AMPA will be retained in those soils.

Oxyfluorfen is a pre- and post-emergence, selective herbicide (U.S. EPA, 2002). It is absorbed by the leaves more readily, especially by the roots of the buds, with little translocation (Rodríguez-Morgado et al., 2014). As can be seen in Figure 3.8c, the herbicide followed the same pattern as GLY: the highest concentration was registered in February 2018, decreasing over time. However, contrary to GLY, this herbicide was not applied in both farms. Even though, according to Ying and Williams (2000), Gómez et al. (2014) and Rodríguez-Morgado et al. (2014), oxyfluorfen has high persistence in soils with a half-life ranging from 72 to 160 days as well as low dissipation rates, the last application of this herbicide was made in March 2017, so it would not be expected to obtain these concentrations after one year. This may indicate that there was an error in the identification of this compound as the analysis was made by an external laboratory. Therefore, these results should be confirmed in the future.

Another herbicide that was detected but not applied was 2,4-D (detected at low concentrations and only in 5 samples out of 6 samples as seen in Table 3.3 and Table 3.4). This herbicide has an estimate half-life between 7 to 312 days depending on the environmental conditions which could explain why it was detected in small concentrations (Magnoli et al., 2020).

Regarding the concentration of fungicides, both farms showed the same pattern: a highest concentration recorded in June 2018 (Figure 3.8d). Within the fungicides detected in the June sampling campaign, the highest mean concentration was observed for dimethomorph, decreasing in QA in the other sampling periods, but remaining similar in QG. Other fungicides detected at highest concentrations were: iprovalicarb and boscalid in QA and metalaxyl and spiroxamine in QG. Indeed, when the sampling took place in June, two applications of dimethomorph have been applied in QA and three in QG; whereas the other fungicides were applied only once. According to our data metalaxyl, despite being detected at highest concentrations in QG, it had not yet been applied when sampling occurred. Similarly, fluopicolide, fluopyram, metrafenone, phthalimide, were not applied, however, they were detected in one or both farms. This could be due to previous applications of these PPPs that resulted in an accumulation of these products in the soil. Indeed, studies have shown that fluopyram has moderate mobility (Chawla et al., 2018) just as it is persistent in soils (Matadha et al., 2020) whereas fluopicolide showed low to medium mobility (NSCFS, 2012). In the case of metrafenone, levels of this fungicide were detected in soil samples prior to fungicide treatment with a concentration of 0.011 ± 0.006 mg kg^{-1} similar to the ones found in this study (Vallejo et al., 2019). Phthalimide is a metabolite with medium to high mobility in soils of folpet which is low persistent in soils with a maximum half-life of 16.2 days (EFSA, 2009), which was applied three times in QA and one in QG before sampling, and its levels in soil samples seem to reflect these differences in frequency of application (Table 3.3 and 3.4). There were also other compounds that were applied in the summer campaign but not detected: metalaxyl and tebuconazole in QA. Other three PPPs (dithianon and fosetyl-aluminum and S) were not analyzed since the methodology used did not allow to quantify these compounds. However, according to their characteristics, it would be expected to find residues from dithianon in the soil samples since it is a fungicide with low mobility in soils whereas fosetyl-aluminum and S would not be detected since the first is not adsorbed to soils and has a short half-life (EFSA, 2005) and S is not persistent in the elemental form and is rapidly transformed into sulfates (EFSA, 2008).

With regard to insecticides, only one was applied (kresoxim-methyl), but it was detected only in one sample from QA (Table 3.3). On the other hand, two compounds were not applied but they were detected: pyrethrin in a limited number of QA samples, for all sampling periods; and indoxacarb in QG also in all sampling periods. This findings are unexpected since these insecticides, indoxacarb and pyrethrin, are non-persistence in soils having relatively short half-life (Antonious, 2003, 2004; Dewey et al., 2012).

It is important to note that the persistence of most of these PPPs is related with the organic matter content as well as other physical and chemical properties of soils which could explain why some of these products were detected in QG but not in QA, especially outside the application period. Moreover, despite the similar pattern observed in the two farms, the highest herbicide concentration recorded in February 2018 and the highest fungicide concentration recorded in June 2018, it is important to note that the number of all PPPs recorded outside of the application period in QG were higher than the ones recorded in QA. This could be explained by the organic matter content that is much higher in QG than QA, being able to retain PPPs as also reported by Sheng et al. (2001) and Sadegh-Zadeh et al. (2017).

3.4.3 Microbial Parameters

Soil's enzymatic activity can be used as a measure of the microbial activity, giving an indication of soil quality and if the microbial activity is being affected by the different management practices (García-Ruiz et al., 2008; Gregorich et al., 1994). Soil enzymes are important for the overall process of organic matter decomposition and nutrients' cycling (Makoi & Ndakidemi, 2008). Soil enzymes are known to be very sensitive to environmental perturbations, such as PPPs application and contamination by potentially toxic elements, which induce quick changes in their activity (Ataikiru et al., 2019; Sanchez-Hernandez et al., 2017; Van Dyk & Pletschke, 2011). Taking this into account, the activity of three enzymes were chosen to be measured in two different sampling periods (February and June 2018), namely, dehydrogenase, arylsulfatase, and acid phosphatase as well as nitrogen mineralization.

Dehydrogenases have an important role on the oxidation of soil organic matter by transferring protons and electrons from substrates to acceptors, as a part of respiration pathways of soil microorganisms. Thus, dehydrogenase activity (DHA) gives an indication of the biological redox-systems as well as a measure on the intensity of microbial metabolism in soil (Gianfreda & Rao, 2010; Schinner et al., 1996). The results obtained in this work showed an increase of DHA between February and June 2018 for both farms (Figure 3.10), even that it was more evident for QG. Several studies were conducted in order to understand how the application of PPPs influence the activity of this enzyme. Wang et al. (2016) in their evaluation of the individual and combined effects of tebuconazole and carbendazim on soil microbial activity, showed that increasing concentrations of moderate and high doses of tebuconazole significantly inhibited DHA. Another study performed by Wang et al. (2017) with the purpose of evaluating the toxicological effects of dimethomorph on soil enzymatic activity and *Eisenia fetida* reported that DHA was significantly inhibited ($p \leq 0.05$) in soils treated with 10 and 100

mg kg^{-1} dimethomorph, concentrations much higher than the ones observed in the present study. However, in the present study, herbicides seemed to be more harmful than fungicides since the DHA activity was much lower in their application period for both farms. In fact, Sebiomo et al. (2011) in their study on the effect of four herbicides on microbial population, soil organic matter and dehydrogenase activity reported that all the herbicides used for treatment in this study resulted in significant reductions in soil DHA when compared to the control. Furthermore, since DHA is directly related to the organic matter (Wolińska & Stępniewska, 2012), the DHA is expected to be higher when there is more organic matter which could explain the differences observed between farms in June 2018. Thus, the results obtained for this enzyme could be mainly attributed to herbicide application as well as organic matter content, however, seasonal variability can also influence the results as also reported by Paz-ferreiro et al. (2011) to which the other sampling periods, that were not yet analyzed, will be crucial to better understand the results.

Arylsulfatase (Aryl) is a soil enzyme that hydrolyzes organic sulfates, making sulfur available for plant uptake (Schinner et al., 1996). This enzyme participates in the sulfur metabolism and its activity is higher when there's lower sulfur availability (Baligar et al., 2005). In this work, the lowest Aryl activity was found in June comparing to February 2018 in both farms. This suggests that, in this case, fungicide application affected more this enzyme than herbicide application. Indeed, Muñoz-Leoz et al. (2011) in their study found lower Aryl activity in tebuconazole-treated soils comparing to the control. Moreover, sulfur-based fungicides were also applied in this period in both farms which could explain the lower activity in this period. Since Aryl activity is higher when there is less sulfur available, it is expected that the addition of this element lowers this enzyme's activity, in a negative feedback mechanism. Nevertheless, seasonal variability, can also influence the results as seen in DHA.

Phosphatases are a group of enzymes very important since they give an indication of soil fertility (Thomsen et al. 2012), play a major role in the phosphorus cycle, affecting plant growth (Makoi & Ndakidemi, 2008; Thomsen et al., 2012). They are released by plant roots and soil microorganisms and produced under conditions of low phosphorus availability (Schinner et al., 1996). The results obtained in this study showed an increase of acid phosphatase (AP) activity between February and June 2018 for both farms (Figure 3.10c), being this increase higher in QG compared to QA. Similarly to DHA, the results could indicate that the application of herbicides had more impact in this enzyme's activity rather than fungicide application. In fact, according to a study conducted by Yu et al. (2011), GLY exhibited a significant inhibitory effect on soil AP activity, even though their study was conducted in a non-cultivated soil. Still, the fact that QA had higher GLY

concentration in February 2018 (Figure 3.8a) could explain the slightly lower AP activity in these soils at that period. Furthermore, Monkiedje and Spiteller (2002) reported that metalaxyl, a fungicide applied in both farms stimulated AP activity in soils. Even though in QA this fungicide was not detected, in QG it was one of the detected PPPs with the highest concentration, especially in June 2018, which may explain why this enzyme's activity was significantly enhanced in QG in June 2018. However, some physical and chemical parameters can also influence the AP activity. For example, some studies showed that AP was influence by the organic matter content (Bhavya et al., 2017; Margalef et al., 2017) as well as by pH_w (Dick et al., 2000; Makoi & Ndakidemi, 2008). Indeed, in this study, AP activity was higher in QG for both sampling periods which had higher OM content. Regarding the pH_w, this chemical parameter seemed to have influenced AP activity since significantly differences between farms in January 2019 were registered for pH_w where QA had lower mean value and low AP activity. Thus, the results obtained in this work can not only be attributed to the application of PPPs but also to the physical and chemical characteristics of the soil. However, as discussed for the past two enzymes, seasonal variability should not be neglected and results for the other sampling periods will be crucial to better understand the results.

Nitrogen mineralization is very important to the nitrogen cycle, since, through processes performed by soils microorganisms, organic nitrogen is converted into the inorganic available form (Schinner et al., 1996; White, 2015). Moreover, the mineralization is directly linked to the organic matter content in the soil since, by decomposing the organic matter, the microorganisms transform the organic N in $NH4^+$ which is latter oxidized into $NO3^-$ (White, 2003). The results obtained for this enzyme's activity were very inconsistent since there was a decrease in activity in QA and an increase in QG between February and June 2018 (Figure 3.10b). The increase observed in QG in June 2018 comparing to QA in that same sampling period might be due to fungicide application, namely due to metalaxyl that was applied in this farm and detected in high concentrations, as also reported by Monkiedje and Spiteller (2002). Regarding the results obtained in QA, it seems that herbicide application enhanced this enzymes activity. Indeed, as showed by Haney et al. (2000) and Haney et al. (2002) the application of GLY had a positive effect in the N mineralization and, since this farm presented higher concentrations of GLY, it is expected that the N mineralization is higher as well. However, as previously mentioned, N mineralization is related to the organic matter content. Taking into account the results obtained regarding the organic matter content the hypothesis arises that the application of PPPs has greater influence in N mineralization than the organic matter content in this case.

3.4.4 Ecotoxicological Assays

I. Aquatic organisms

Allivribio fischeri bioluminescence assays have been widely used in ecotoxicology with the purpose of investigating the acute effects of micropollutants onto the aquatic environment, due to their reliability and sensitivity to several contaminants (Kungolos, Emmanouil, Tsiridis, & Tsiropoulos, 2009). Since the results obtained for this assay did not allow the determination of EC_{10}, EC_{20} or EC_{50}, the observed effect at the highest concentration and after 30 minutes of exposure was registered. In this study, toxicity was considered when the effect was above 20% (Baran & Wieczorek, 2015; Persoone et al., 2003). Taking this into account, toxicity was only registered in 2 QA and 1 QG samples in February and April 2018, 2 QA and 3 QG samples in June 2018 and 2 QA and 4 QG samples in January 2019 (Table 3.6). Some studies have reported that the greater the potentially toxic elements concentration and the PPPs application, the greater the toxicity to this aquatic organism (Aruoja et al., 2015; Gälli et al., 1994; Ruiz et al., 1997; Yang et al., 2016). However, taking into account the results obtained in this work for the potentially toxic elements levels, this does not seem the explanation for the toxicity observed. Even though some studies reported toxicity to this bacteria due to fungicide application (Kungolos et al., 2009; Westlund et al., 2018; Yang et al., 2011), as can be seen in Table 3.6 the application of fungicides and herbicides does not seem to have negative effects to this aquatic organism in this study as the highest effect recorded for Microtox® is not accompanied by the highest herbicide/fungicide concentration.

The algae community is essential for the aquatic ecosystem since they are responsible for the production of oxygen and organic substances which are essential for other aquatic organisms (Bérard, 1996). Therefore, chemical effects on algae can directly affect the structure and function of an ecosystem, leading to oxygen depletion or decreased primary productivity (Campanella et al., 2001; Wong, 2000). This demonstrates the importance of including algae growth inhibition assays in the evaluation of potential adverse effects from PPPs application. The results presented demonstrated that the highest inhibition was registered in February 2018 (Figure 3.11) when herbicides were applied decreasing in the following months in both farms. Indeed, the concentration of glyphosate (GLY) and oxyfluorfen was also higher in February 2018 decreasing in the following sampling periods being this concentration higher in QA than QG (Figure 3.8a,c). Thus, the growth inhibition of this algae is likely to be related with the presence of herbicides. The same effect was observed in a study conducted by Ma et al. (2006) in which the toxicity of 40 herbicides was assessed in this algae, demonstrating that GLY negatively affected algae growth. As for oxyfluorfen, the

inhibition of algae growth was also recorded in *Scenedesmus obliquus* exposed to 5, 10, 20, and 30 µg L^{-1} (Geoffroy et al., 2002) and 7.5, 15, and 22.5 µg L^{-1} (Geoffroy et al., 2003).

Lemna minor is a floating aquatic macrophyte and a natural component of many aquatic ecosystems. This macrophyte has been widely used in ecotoxicology to investigate the acute effects of micropollutants onto the aquatic environment, due to its small size, fast growth rate, vegetative reproduction, reliability and sensitivity to several pollutants (Wang, 1990). In this study, the higher *L. minor* growth inhibition was registered in January 2019 in QA and June 2018 in QG for number of fronds (Figure 3.12a) whereas for the dry weight the highest inhibition was obtained in June 2018 in QA and QG (Figure 3.12b). Since *L. minor* is an aquatic plant, the application of herbicides was expected to be more harmful to this species, however overall, the growth of this organism seemed to be more affected by fungicide application. However, there is not much information on the effects of fungicides, as the ones applied in these farms, on *L. minor*. Still, in a review by Zubrod et al. (2019) and in a study performed Marinho et al. (2020), the fungicides such as myclobutanil, cymoxanil, and azoxystrobin had negative effects in *L. minor* growth.

Taking this into account, the analysis of all the evidence provided by the aquatic tests indicates that the present levels of PPPs affected the soil retention function, since they are being mobilized for the aquatic fraction, causing harmful effects in the tested organisms.

II. Terrestrial organisms

Earthworms are commonly used in ecotoxicological assessment since they are important members of the soil community due to their ability to change their habitat as well as create new habitats for other organisms (Römbke et al., 2005). The avoidance test is a fast and useful screening tool for soil contamination since earthworms are exposed to them in both the aqueous and solid phase of the soil compartment either by soil ingestion or dermal contact (Antunes et al., 2008; Römbke et al., 2005; Schaefer, 2004). This, allied with the existence of chemoreceptors and sensory tubercles in these organisms, gives them a high sensibility to chemicals in soils which may contribute to the greater sensitivity of avoidance assays (Reinecke et al., 2002). Thus, the assays carried out with these soil organisms allowed to verify if the habitat function of farm soils was compromised with PPPs application. The avoidance assay results (Figure 3.13) of the present study indicate that the habitat function of soils in the three sampling periods for both farms was not compromised since the avoidance recorded, even though not statistically significant, demonstrates that the test soil has recovered to the quality of

January 2019 in terms of habitat function. This suggests that the concentrations of PPPs found in these soils, were not high enough to completely compromise this relevant function of vineyard soils. Similarly, in a study conducted by Santos et al. (2012) with the objective of evaluating the effects of three PPPs, after application on an agricultural field on the avoidance behavior and reproduction output of *Eisenia andrei* and *F. candida*, the results showed non avoidance of earthworms in field recommended doses of Montana® (GLY 308 g L^{-1} a.i.). The same results were found by Niemeyer et al. (2018) in their work with the aim of evaluating the effects of four commercial GLY herbicides on soil invertebrates in which non-significant response were recorded in the avoidance assay with *E. andrei*. Regarding fungicide application, Rico et al. (2016) in their investigation of the toxicity of five PPPs typically used in rice farming on the earthworm *E. fetida* found no significant avoidance behavior for tebuconazole having even observed a slight attraction though not significant when compared to the controls.

In viticulture, one way to increase soil's biodiversity is to use cover plants such as *Medicago sativa*. This plant can be used as soil cover between the rows of vine plants, being very important to prevent erosion and leaching of nutrients in agroecosystems (Büchi et al., 2018). *M. sativa* is known by its ability to improve both soils' structure and biochemical activity (Hamdi et al., 2012), however, even though they are not intentionally treated, these non-target plants can be affected by PPPs application. The results obtained in this study demonstrated that for all the parameters measure in the seedling emergence and seedling growth test, the plants that grew in QA soil were more affected than the one from QG soil (Figures 3.13 and 3.14). The fact that QG had higher values for % germination and survival as well as for root and shoot length and fresh biomass can be related to the organic matter levels found in that farm, that were much higher than QA (Figure 3.5d). In fact, one of the important roles of organic matter is to store and supply nutrients such as nitrogen, phosphorus, and sulfur, which are needed for the growth of plants (USDA, 1996). Moreover, high OM content might reduce the bioavailability of contaminants as previously discussed.

Looking to the different sampling periods, the percentage of germination was significantly lower than the control group in February 2018 for QA soil samples (Figure 3.13a). This suggests that concentration of herbicides might be the reason for this negative affect (Figure 3.8a,b,c). For QG there was also a slightly decrease in % germination between the control and February 2018 even though it was not statistically different (Figure 3.13b), reflecting the lower herbicides concentrations found in these soil samples comparing with QA (Figure 3.8). The reduced seed production and emergence caused by GLY application was reported by Clay and Griffin (2000) in their study with *Sesbania exaltata*, *Senna obtusifolia* and *Xanthium strumarium* at a rate of 0.42 kg ha^{-1}

(much lower than the dose of 1.44 kg ha^{-1} applied in this study) and by Gomes et al. (2017b) in *Dimorphandra wilsonni* seeds submitted to different GLY concentration (0, 5, 25 and 50 mg active ingredient L^{-1}). Another important indicator of this essay is the % of survival because, even though all seeds may have germinated, some died during the essay. In this case, QG was hardly affected whereas QA was most affected in February 2018 comparing with the other sampling periods (Figure 3.13). Again, herbicide concentration seemed to have had a greater impact on this indicator in QA.

Regarding root length and fresh biomass (3.14a,b), these endpoints showed a decrease in February and June 2018 for both farms, being the difference statistically significant only for QA. According to these results, it is possible to note that these negative effects might be due to PPPs application, however, there seems to be a recovery in the next sampling periods as root length and fresh biomass increased in April 2018 and January 2019. As for shoot length and biomass (3.14c,d), there was a significant decrease between the control group and February and April 2018 in QA, recovering in the following periods. On the other hand, for QG no significant differences between the sampling periods and the control group were observed, despite a slightly decrease in June 2018 and an increase in January 2019. In the laboratory experiment conducted in Chapter 2, a cover crop was exposed to increasing concentrations of a GLY-based herbicide. A reduction of root and shoot length and biomass was recorded being significant at the lowest concentration tested (8 mg kg^{-1}) for shoot fresh biomass. As discussed in that chapter, several studies were conducted in order to understand the effects that GLY application had on plant growth all of which reported a decrease in the plant's biometric parameters (Basantani et al., 2011; Debski et al., 2018; Mondal et al., 2017; Singh et al., 2017; Singh et al., 2017; Soares et al., 2019; Spormann et al., 2019). Even though a significant decrease in plant growth was registered in this study in February 2018, the results can not only be attributed to GLY as other herbicides (oxyfluorfen and 2,4-D) were detected. As for the differences observed between farms, these may be attributed to the OM content since higher values of root and shoot growth and fresh biomass were observed in QG which has higher OM content. Regarding fungicide application, since these products are used to eliminate or prevent the growth of fungi and their spores, it was not expected to observe negative effects in non-target plants due to its use. Nevertheless, fungicide application seemed to affect this plants' biometric parameters. However, as well as for the *L. minor* growth inhibition assay, there is not much information on the effects of fungicides on cover crops.

4 Conclusions

4.1 Conclusions

With this work, it was possible to assess the effects of soil contamination by a GLY-based herbicide (GBH) on the growth and redox homeostasis of a cover plant species, to evaluate whether the application of plant protection products (PPPs) in the Douro Demarcated Region (DDR) vineyards compromises soil quality and to verify if the soils recovered at the end of each annual cycle of phytopharmaceutical treatments. For the first main objective, presented in Chapter Two, an artificial soil was contaminated with increasing concentrations of a commercial GLY-based herbicide, to which seeds of *Medicago sativa* were added in laboratory conditions. It is possible to conclude that after 21 days of exposure, the growth and physiological performance of *M. sativa*, were negatively affected at the concentrations tested. The results also showed an activation of the AOX system, although its action was not enough to counteract the oxidative damage induced by an overproduction of ROS, ultimately leading to a decrease in this plant's growth. In the present work adverse effects of GLY were visible at 8 mg kg^{-1} of the a.i., which is a concentration much lower than the highest levels reported for European and South American soils, but much higher than the mean values observed in the Douro vineyard soils that are presented in Chapter Three. It should be noted that soil properties such as soil organic matter content, may affect the behavior of GLY on soils, as well as the type of formulation that can also affect the toxicity, since the presence of surfactants may enhance the negative effects of the a.i. Thus, considering that plant responses to GLY can be species-specific and vary with distinct experimental conditions, it is of upmost importance to better understand the impacts of GLY-contaminated soils on the survival of non-target plants and subsequently on soils biodiversity, as well as developing new strategies to minimize its potential risks to agroecosystems.

The field study conducted (Chapter Three), allowed us to understand the effects of PPPs on soil quality, as well as assess the differences between sampling periods providing a comprehensive view of the quality of soils through the evaluation of physical, chemical, biological and ecotoxicological indicators. However, the assessment of these indicators is a very difficult process due to the complexity of the system as well as the interdependence of several parameters. Regarding the physical and chemical indicators, only the pH seems to be affected by the fungicide application such as the ones based on sulfur. The differences observed between the two farms for some of these indicators such as OM content and major elements concentrations are likely to be related with the soil's natural properties together with some differences in the management practices. As for PPPs concentrations in soils, the levels of GLY were, as expected, higher in the period of herbicide application in both farms, whereas AMPA concentrations remained

similar. Regarding the rest of the PPPs analyzed, the products that were applied and detected showed to be within the concentrations found in vineyard soils, however, the most concerning fact was the detection of PPPs that were not applied throughout the sampling period, as well as the similar levels of applied fungicides within sampling periods. This suggested that these products were accumulated in soils, especially is soils with higher content of OM, mostly due to their persistence, which highlights the need to monitor the concentrations over time. Soil quality was also assessed by the enzymatic activity of soils as well as by the nitrogen mineralization. Herbicide application appears to have affected the dehydrogenase activity whereas fungicide application seems to be responsible for the results obtained for acid phosphatase and arylsulfatase activities. However, the enzyme's activity and nitrogen mineralization could also be affected by soil physical and chemical characteristics such as organic matter content and pH as well as seasonal variation which will be evaluated by assessing the enzymatic activity in other sampling periods. Nevertheless, microbial activity proved to be a good parameter to assess the soil quality, being sensitive to the application of PPPs. Impacts on the functioning of the soil microbial community may contribute as well, in the long-term, for the increasing presence of PPPs residues in soils.

Ecotoxicological assays are an important tool to assess whether PPPs application affects soil functions such as retention of contaminants and habitat provision. The retention of contaminants is relevant for all the soils but also for those found in the DDR, with vines implemented in steep slopes which enhances the dispersion of contaminants that can be leached and contaminate surface and groundwater. Thus, the ecotoxicological assays with aquatic organisms showed that this function is being affected since *Raphidocelis subcapitata* growth was impaired by herbicide application whereas *Lemna minor* seemed to be more affected by fungicide application rather than herbicide application. However, habitat function did not seem to be affected, as the avoidance assay with *Eisenia fetida* showed no significantly avoidance towards the contaminated soil. Moreover, the contaminated soils showed levels of habitat provision similar to those offered at the end of the herbicide application period. Lastly, primary production, a service provided by soils, seemed to be affected by herbicide and fungicide application as *Medicago sativa* growth parameters significantly decrease in those sampling periods.

It is important to note that, even though soil quality seemed to be affected by PPPs in the moment of application, the system seems to be able to recover from this application over time. In the future, it would be interesting to evaluate the levels of the available elements / nutrients, in addition to the pseudo-total concentration since these results could help understand the ecotoxicological results. Furthermore, other soil enzymes

should be analyzed namely cellulase, urease and the potential nitrification that are involved in the carbon cycle, hydrolysis of urea fertilizer and oxidation of ammonium (NH_4^+) or ammonia (NH_3) to nitrate, respectively being important to understand if the nutrient cycling is in fact being affected (Makoi & Ndakidemi, 2008; Sahrawat, 2008). In addition, soil microbial biomass would be an interesting indicator to measure. More ecotoxicological assays should also be performed with other aquatic species such as *Daphnia magna* and terrestrial organisms such as *Folsomia candida* to further investigate if soil functions are affected by PPPs application. The acquisition of more data will increase the lines of evidence for a more comprehensive and robust understanding of the impact of production modes on the quality of vineyard soils.

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Annex

Table A1. Characteristics of inorganic and organic fertilizers applied in both farms.

Type	YARA MILA SOLAN 13-11-21	PHENIX 6-8-14
N%	13 (5.3 NO_3 + 7.7NH_3)	6
P_2O_5%	11	8
K_2O%	21	15
CaO%	-	5
MgO%	2	3
B%	0.2	-
TOC%	-	29
MO%	-	50
C/N	-	5
pH	-	6-7

Table A2. Statistical analysis of chemical and physical parameters measured in the two farms studied in two sampling periods.

Parameters	Farm	Sampling period	Mean	STD	CV (%)	Median	Min	Max
pH_w	QA	Jun/18	6.05	0.23	3.86	6.07	5.69	6.38
		Jan/19	6.40	0.22	3.38	6.38	6.15	6.69
	QG	Jun/18	5.80	0.25	4.29	5.82	5.39	6.08
		Jan/19	6.06	0.19	3.18	6.09	5.83	6.26
pH_{KCl}	QA	Jun/18	6.08	0.22	3.54	6.03	5.83	6.33
		Jan/19	6.10	0.29	4.72	6.02	5.69	6.49
	QG	Jun/18	5.08	0.27	5.39	5.17	4.66	5.41
		Jan/19	5.30	0.22	4.09	5.31	5.04	5.55
EC ($\mu S\ cm^{-1}$)	QA	Jun/18	84.41	40.67	48.19	75.55	47.70	171.4
		Jan/19	122.6	53.57	43.70	107.1	55.70	220.3
	QG	Jun/18	113.0	60.28	53.34	92.80	59.70	190.6
		Jan/19	71.53	25.12	35.12	64.05	45.55	110.0
OM (%)	QA	Jun/18	1.839	0.8638	46.98	2.150	0.590	2.660
		Jan/19	1.929	0.5869	30.43	1.630	1.240	2.640
	QG	Jun/18	3.957	1.190	30.07	3.765	2.690	5.500
		Jan/19	3.683	0.9491	25.77	3.695	2.120	4.860

Table A3. Descriptive statistic of the concentration of major elements in June 2018 and January 2019 in both farms studied.

Major elements (mg kg^{-1})	Farm	Sampling period	Mean	STD	CV (%)	Median	Min	Max
Al	QA	Jun/18	10023	2073	20.68	9116	8593	14531
		Jan/19	9384	563.1	6.001	9434	8636	14531
	QG	Jun/18	16877	1437	8.513	17316	14812	18293
		Jan/19	18889	1840	9.744	19744	16283	20446
Fe	QA	Jun/18	34618	2321	6.704	33605	31912	37380
		Jan/19	34774	1302	3.744	35097	32769	36156
	QG	Jun/18	44586	3146	7.057	44871	40017	49432
		Jan/19	42372	3049	7.197	42692	38776	45440
Mg	QA	Jun/18	8160	444.1	5.443	8023	7700	8814
		Jan/19	8291	395.7	4.772	8469	7597	8731
	QG	Jun/18	10535	960.8	9.121	10659	9108	11459
		Jan/19	10331	1128	10.92	10358	8969	11651
P	QA	Jun/18	653,5	226,0	34,58	576,5	503.3	1158
		Jan/19	660.6	137.8	20.86	617.6	489.7	921,6
	QG	Jun/18	498.2	103.6	20.79	489.6	370.4	670.7
		Jan/19	503.2	51.60	10.25	508.0	441.4	576.3
K	QA	Jun/18	4082	786.1	19.26	3835	3291	5653
		Jan/19	4309	1022	23.72	3759	3149	5902
	QG	Jun/18	6147	715.2	11.63	6276	4890	6930
		Jan/19	6163	835.7	13.56	6301	5069	6924
Ca	QA	Jun/18	3583	725,1	20,24	3728	2623	4451
		Jan/19	3585	661.8	18.46	3149	2984	4543
	QG	Jun/18	2210	537.3	24.32	2153	1649	3208
		Jan/19	2154	334.8	15.54	2116	1791	2703
Mn	QA	Jun/18	340.5	37.22	10.93	345.9	279.2	382.2
		Jan/19	326.9	39.46	12.07	331.0	282.8	387.8
	QG	Jun/18	343.0	66.02	19.25	341.5	239.8	445.1
		Jan/19	315.5	41.99	13.31	331.0	231.8	342.9

Table A4. Results obtained for the Two-Way Anova for the major elements with significant statistical differences between farms/sampling periods.

Major elements	Source of variation	Sampling period	Test	t	df	p
Fe	Farm	Jun/18	Unpaired t-test	6.570	11	<0.05
		Jan/19	Unpaired t-test	6.017	11	<0.05
Mg	Farm	Jun/18	Unpaired t-test	5.878	11	<0.05
		Jan/19	Unpaired t-test	4.499	11	<0.05
K	Farm	Jun/18	Unpaired t-test	4.920	11	<0.05
		Jan/19	Unpaired t-test	3.539	11	<0.05
P	Farm	Jan/19	Unpaired t-test	2.630	11	<0.05
Ca	Farm	Jun/18	Unpaired t-test	3.818	11	<0.05
		Jan/19	Unpaired t-test	4.778	11	<0.05

Table A5. Descriptive statistic of the concentration of trace elements in June 2018 and January 2019 in both farms studied.

Trace elements (mg kg^{-1})	Farm	Sampling period	Mean	STD	CV (%)	Median	Min	Max
Cu	QA	Jun/18	67.02	8.013	11.96	67.41	56.30	77.28
		Jan/19	68.83	12.57	18.26	69.28	53.56	83.46
	QG	Jun/18	55.17	16.61	30.10	52.99	36.40	81.73
		Jan/19	55.98	11.81	21.09	52.51	42.17	77.32
Zn	QA	Jun/18	98.58	23.66	24.00	88.20	82.91	145.1
		Jan/19	101.1	23.11	22.87	96.66	76.86	141.7
	QG	Jun/18	92.99	8.757	9.418	95.71	79.81	101.3
		Jan/19	95.57	8.591	8.989	96.09	84.73	106.7
Ni	QA	Jun/18	26.64	2.049	7.693	26.46	24.59	29.64
		Jan/19	26.37	1.624	6.159	27.16	24.15	27.95
	QG	Jun/18	29.64	3.277	11.06	29.55	26.43	35.29
		Jan/19	29.89	3.511	11.75	29.59	25.91	35.36
V	QA	Jun/18	24.58	1.111	4.519	24.32	23.36	26.49
		Jan/19	24.22	1.171	4.837	23.94	23.08	26.15
	QG	Jun/18	32.32	2.364	7.314	31.97	30.12	36.41
		Jan/19	30.29	2.765	9.132	29.52	27.52	34.67

Table A5 (Continuation). Descriptive statistic of the concentration of trace elements in June 2018 and January 2019 in both farms studied.

Trace elements (mg kg^{-1})	Farm	Sampling period	Mean	STD	CV (%)	Median	Min	Max
Cr	QA	Jun/18	31.98	3.786	11.84	30.78	28.23	37.49
		Jan/19	33.36	4.279	12.83	33.15	28.74	41.78
	QG	Jun/18	40.06	2.530	6.317	40.04	36.90	43.72
		Jan/19	38.56	2.717	7.046	38.00	35.47	42.34
Co	QA	Jun/18	15.92	1.439	9.036	15.92	14.39	18.00
		Jan/19	15.77	0.9102	5.772	15.71	14.14	16.90
	QG	Jun/18	17.94	1.927	10.74	17.61	16.06	20.94
		Jan/19	16.99	2.230	13.13	16.36	15.13	21.36
As	QA	Jun/18	9.486	11.27	118.8	5.480	2.200	34.70
		Jan/19	5.706	4.560	79.93	5.440	1.840	15.41
	QG	Jun/18	8.462	1.901	22.46	8.205	6.600	10.66
		Jan/19	8.450	2.102	24.88	8.155	5.570	11.12
Cd	QA	Jun/18	0.2757	0.4191	152.0	0.1200	0.0700	1.220
		Jan/19	0.1229	0.0789	64.21	0.0900	0.0500	0.290
	QG	Jun/18	0.1800	0.0930	51.64	0.1450	0.1100	0.350
		Jan/19	0.1367	0.0393	28.78	0.1300	0.0900	0.200
Ba	QA	Jun/18	41.08	7.457	18.15	42.17	32.24	53.71
		Jan/19	40.63	4.614	11.36	39.68	35.91	50.19
	QG	Jun/18	53.41	8.083	15.13	54.14	41.12	63.50
		Jan/19	57.61	15.28	26.52	55.61	42.75	84.83
Pb	QA	Jun/18	12.26	3.320	27.08	12.05	7.940	17.21
		Jan/19	12.71	4.152	32.66	11.20	9.410	18.81
	QG	Jun/18	20.95	5.906	28.20	17.63	16.71	29.83
		Jan/19	16.92	2.591	15.32	17.07	12.45	20.07

Table A6. Results obtained for the Two-Way Anova for the trace elements with significant statistical differences between farms/sampling periods.

Trace elements	Source of variation	Sampling period	Test	t	df	p
Ni	Farm	Jan/19	Unpaired t-test	2.359	11	<0.05
V	Farm	Jun/18	Unpaired t-test	7.757	11	<0.05
		Jan/19	Unpaired t-test	5.308	11	<0.05
Cr	Farm	Jun/18	Unpaired t-test	4.431	11	<0.05
		Jan/19	Unpaired t-test	2.560	11	<0.05
Ba	Farm	Jun/18	Unpaired t-test	2.860	11	<0.05
		Jan/19	Unpaired t-test	2.812	11	<0.05
Pb	Farm	Jun/18	Unpaired t-test	3.339	11	<0.05

Table A7. List of plant protection products analyzed by LC-MS.

LAB 1-01-128 MR NORMAL			
Abamectin	Chloroxuron	Dinotefuran	Flufenacet
Acephate	Chlorpropham	Dinoterb	Flufencin
Acetamiprid	Chlorsulfuron	Disulfoton	Fluometuron
Acibenzolar-S-methyl	Chromafenozide	Disulfoton sulfone	Fluopicolide
Acibenzolar acid	Cinosulfuron	Disulfoton sulfoxide	Fluopyram
Aldicarb	Clethodim	Diuron DMSA	Fluoxastrobin
Aldicarb sulfone	Climbazole	DMST	Fluquinconazole
Aldicarb sulfoxide	Clofentezine	Dodemorph	Flurochloridrone
Ametoctradin	Clomazone	Dodina	Fluroxypyr
Ametryn	Clothianidin	Emamectin	Flurprimidol
Aminocarb	Coumaphos	EPN	Flurtamone
Amisulbrom	Cyantraniliprole	Epoxiconazole	Flusilazole
Amitraz	Cyanazine	Ethiofencarb	Flutolanil
Anilophs	Cyazofamid	Ethiofencarb-sulfone	Flutriafol
Atrazine	Cycloxydim	Ethiofencarb-sulfoxide	Forchlorfenuron
Atrazine-desethyl	Cyflumetofen	Ethiprole	Formetanate
Atrazine-diisopropyl	Cyhexatin	Ethirimol	Fosthiazate
Azaconazole	Cymoxanil	Ethofumesate	Fuberidazole
Azadirachtin	Cyprodinil	Etofenprox	Haloxyfop
Bendiocarb	Cyromazine	Etoxazol	Haloxyfop-ethoxyethyl
Bensulfuron-methyl	2,4 D	Famofos	Haloxyfop-methyl
Bentazone	DEET	Fenamidone	Hexythiazox
Benthiavalicarb-isopropyl	Demeton-S-methyl sulfone	Fenbuconazole	3-hydroxycarbofuran
Bifenazate	Desethyl terbumeton	Fenbutatin oxide	Imazalil
Bioallethrin	Desethyl-terbuthylazine	Fenhexamide	Imazamox
Bixafen	Desmedipham	Fenoxaprop-P-ethyl	Imazapyr
Bromoxynil	Desmethrin	Fenpiclonil	Imidacloprid

Table A7. (Continuation) List of plant protection products analyzed by LC-MS.

LAB 1-01-128 MR NORMAL			
Bromuconazole	Iprovalicarb	Penoxsulam	Spirotetramat
Butocarboxim	Isoprocarb	Penthiopyrad	Spirotetramat-enol
Butocarboxim-sulfoxide	Isoproturon	Pethoxamid	Spirotetramat-enol-glucoside
Butoxicarboxim	Isoxaben	Phenmedipham	Spirotetramat-ketohydroxy
Carbaryl	Isoxaflutole	Phenpropidine	Spirotetramat-monohydroxy
Carbendazim + benomyl	Lenacil	Phorate	Spiroxamine
Carbetamide	Linuron	Phorate-sulfone	Sulcotrione
Carbofuran	Malaoxon	Phorate-sulfoxide	Tebufenozide
Carboxin	Mandipropamid	Phosmet oxon	Tebufenpyrad
Carfentrazone-ethyl	Mecarbam	Phosphamidon	Tebutam
Carpropamide	Mepanipyrim	Phoxim	Teflubenzuron
Chlorantranilipole	Mepanipyrim-2-hidroxypropyl	Pinoxaden	Temefos
Chloridazon	Mepronil	Piperonyl-butoxide	Tepraloxydim
Chlorotoluron	Mesotrione	Piraflufene-ethyl	Terbufos
Diallate	Methabenzthiazuron	Pirasulfutole	Terbumeton
2,6-Dichlorobenzamide	Metaflumizone	Pirimicarb	Terbuthylazine
Dichlofluanid	Metamitron	Pirimicarb-dimethyl	Thiabendazole
Dichlorprop	Metazachlor	Profoxidim	Thiacloprid
Dichrotophos	Metconazole	Promecarb	Thiamethoxam
Diclobutrazol	Methacryphs	Promethrin	Thiazopyr
Diethofencarb	Methiocarb	Propamide	Thifensulfuron-methyl
Diflubenzuron	Methiocarb sulfone	Propamocarb	Thiocyclam
Dimefuron	Methiocarb sulfoxide	Propaquizafop	Thiodicarb
Dimethachlor	Methomyl	Propetamphos	Thiofanox
Dimethenamid	Methoxyfenozide	Propoxycarbazone	Thiofanox-sulfone
Dimethoate	Metobromuron	Proquinazide	Thiofanox-sulfoxide
Dimoxystrobin	Metolachlor	Prosulfocarb	Thiophanate-methyl
Diniconazole	Metolcarb	Prosulfuron	TNFA
Fenpropimorph	Metosulam	Prothioconazole-desthio	TNFG
Fenpyrazamine	Metoxuron	Pymetrozine	Tolylfluanid
Fenpyroximate	Metrafenone	Pyraclostrobin	Tralkoxydim
Fenpropimorph	Metribuzin	Pyroxsulam	Triadimefon
Fensulfothion	Metsulfuron-methyl	Quinclorac	Triadimenol
Fenthion-oxon	Molinato	Quinmerac	Triallate
Fenthion-oxon-sulfone	Monocrotophos	Quinoxyphen	Triasulfuron
Fenthion-oxon- sulfoxide	Monolinuron	Quizalofop	Triazophos
Fenthion sulfone	Monuron	Quizalofop-ethyl	Trichlorfon
Fenthion sulfoxide	Napropamide	Rotenone	Triclopyr
Flonicamid	Neburon	Sebutilacin	Tricyclazole
Fluazifop	Nicosulfuron	Sethoxydim	Tricresyl-phosphate
Fluazifop-P-butyl	Nitempiram	Silthiofam	Trietazine
Fluazinam	Ofurace	Simazine	Trifloxystrobin
Flubendiamide	Omethoate	Simetryn	Triflumizole
Indoxacarb	Oryzalin	Spinetoram	Triflumuron
Iodosulfuron-methyl	Oxadiargyl	Spinosad	Vamidothion
Ioxynil	Oxamyl	Spirodiclofen	Zoxamide

Table A8. List of plant protection products analyzed by GC-MS.

LAB 1-01-80 MR NORMAL GASES			
Aclonifen	Dicloran	Heptachlor-epoxide endo	Penconazole
Acrinathrin	1,1-dichloro-2,2-bis(4-ethylphenil)ethane(Pertano)	Heptachlor-epoxide exo	Pendimethalin
Alachlor	Dichlorvos	Heptenophos	Pentachloroaniline
Aldrin	Dieldrin	Hexachlorocyclohexane alfa	Pentachloroanisole
Azoxystrobin	Difenoconazole	Hexachlorocyclohexane beta	Permethrin
Benalaxyl (including Benalaxyl-M)	Diflufenican	Hexachlorocyclohexane delta	Phenothrin
Benfluralin	Dimethomorph	Hexaconazole	Phenthoate
Bifenox	Diphenamide	Hexazinone	2-phenylphenol
Bifenthrin	Diphenylamine	Iodofenphos	Phosmet
Boscalid	Ditalimfos	Iprodione	Piridafention
Bromacil	Endosulfan alfa	Isocarbophos	Pirimiphos-ethyl
Bromophos	Endosulfan beta	Isofenphos	Pirimiphos-methyl
Bromophos-ethyl	Endosulfan sulphate	Isofenphos-methyl	Prochloraz
Bromopropylate	Endrin	Isopropaline	Procymidone
Bupirimate	Ethion	Isoprothiolane	Profam
Buprofezin	Ethoprophos	Kresoxim-methyl	Profenofos
Butralin	Etridiazole	Lambda-Cyhalothrin	Profluralin
Cadusafos	Etrimfos	Leptophos	Propachlor
Carbofenotion	Famoxadone	Lindane	Propanil
Chlorbenside	Fenpropathrin	Malathion	Propargite
Chlordane	Fenamiphos	Metalaxyl (including Metalaxyl-M)	Propiconazole
Chlorfenapyr	Fenamiphos sulfone	Methamidophos	Propoxur
Chlorfenson	Fenamiphos sulfoxide	Methidathion	Prothiofos
Chlorfenvinphos	Fenchlorphos	Mevinphos	Pyrazophos
Chloroneb	Fenitrothion	Mirex	Pyridaben
Chloropropylate	Fenoxycarb	Myclobutanil	Pyridalyl
Chlorpyrifos	Fenson	Nitrofen	Pyrifenox
Chlorpyrifos-methyl	Fenthion	Nitrothal isopropyl	Pyrimethanil
Chlorthal dimethyl	Fenvalerate + Esfenvalerate	Norflurazon	Pyriproxyfen
Chlozolinate	Fipronil	Nuarimol	Quintozene
Clodinafop-propargyl	Fipronil sulfone	o,p-DDD	Silafluofen
Crimidine	Flucitrinate	o,p-DDE	Sulfotep
Cyanofenphos	Fludioxonil	o,p-DDT+p,p'-TDE (DDD)	Sulprofos
Cycloate	Flumioxazin	Oxadiazon	Tau fluvalinate
Cyflufenamid	Fluotrimazole	Oxadixyl	Tebuconazole
Cyfluthrin	Fonofos	Oxyfluorfen	Tecnazene
Cypermethrin	Formotion	p,p'-DDE	Tefluthrin
Cyproconazole	Fosalon	p,p'-DDT	Terbutryn
Deltamethrin	Furalaxil	Paraoxon-methyl	Tetrachlorvinfos
Diazinon	Furathiocarb	Parathion	Tetraconazole
Dichlofenthion	Heptachlor	Parathion-methyl	Tetradifon

Table A8. (Continuation) List of plant protection products analyzed by GC-MS.

LAB 1-01-80 MR NORMAL GASES
Thiobencarb
Tolclofos-methyl
Transfluthrin
2,4,6-Trichlorophenol
Trichloronate
Trifluralin
Vinclozolin

Table A9. Descriptive statistics of the concentration of glyphosate and AMPA in the four sampling periods in both farms studied.

	Farm	Sampling period	Mean	STD	CV (%)	Median	Min	Max
Gly (mg kg^{-1})	QA	Feb/18	2.78	3.03	109	2.06	0.0700	8.50
		Apr/18	0.563	0.424	75.2	0.595	0.100	1.24
		Jun/18	0.668	0.837	125	0.225	0.0600	2.03
		Jan/19	0.139	0.096	69.0	0.130	BDL	0.250
	QG	Feb/18	1.47	0.56	38.0	1.58	0.780	2.01
		Apr/18	0.748	0.453	60.6	0.805	0.170	1.27
		Jun/18	0.385	0.463	120	0.170	0.0500	1.27
		Jan/19	0.163	0.117	71.8	0.125	BDL	0.350
AMPA (mg kg^{-1})	QA	Feb/18	0.626	0.309	49.4	0.550	0.330	1.29
		Apr/18	0.692	0.294	42.6	0.630	0.410	1.22
		Jun/18	0.607	0.315	51.9	0.495	0.310	1.11
		Jan/19	0.760	0.182	23.9	0.820	0.440	1.00
	QG	Feb/18	1.21	0.50	41.8	1.11	0.640	2.04
		Apr/18	1.38	0.54	39.6	130	0.860	2.31
		Jun/18	1.02	0.63	62.0	0.775	0.430	2.14
		Jan/19	0.967	0.411	42.5	0.795	0.580	1.61

BDL: below the detection limit

Table A10. Descriptive statistics of the concentration of the detected plant protection products in the four sampling periods for both farms studied.

PPP	Farm	Sampling period	Mean	STD	CV (%)	Median	Min	Max
Boscalid (mg kg^{-1})	QA	Feb/18	0.011	0.006	54.8	0.009	0.006	0.020
		Apr/18	0.012	0.007	55.7	0.011	0.006	0.020
		Jun/18	0.031	0.010	31.5	0.030	0.018	0.046
		Jan/19	0.040	0.027	68.2	0.034	0.012	0.087
	QG	Feb/18	0.022	0.017	76.3	0.020	0.006	0.044
		Apr/18	0.017	0.013	72.6	0.016	0.006	0.030
		Jun/18	0.014	0.0074	51.4	0.017	0.006	0.020
		Jan/19	0.019	0.014	73.9	0.016	0.007	0.035
Dimethomorph (mg kg^{-1})	QA	Feb/18	0.163	0.059	36.4	0.155	0.098	0.260
		Apr/18	0.102	0.062	60.5	0.091	0.040	0.220
		Jun/18	0.352	0.178	50.7	0.350	0.16	0.560
		Jan/19	0.172	0.078	45.3	0.170	0.089	0.2900
	QG	Feb/18	0.188	0.118	62.6	0.130	0.072	0.330
		Apr/18	0.131	0.058	44.0	0.110	0.085	0.230
		Jun/18	0.180	0.063	35.1	0.190	0.110	0.270
		Jan/19	0.148	0.080	53.5	0.130	0.083	0.280
Fluopicolide (mg kg^{-1})	QA	Feb/18	0.10	0.037	37.0	0.10	0.039	0.15
		Apr/18	0.058	0.016	28.0	0.055	0.040	0.086
		Jun/18	0.038	0.010	26.8	0.040	0.023	0.049
		Jan/19	0.023	0.007	32.0	0.022	0.014	0.033
	QG	Feb/18	0.008	-	-	0.008	0.008	0.008
		Apr/18	0.006	0.0014	23.6	0.006	0.005	0.007
		Jun/18	-	-	-	-	-	-
		Jan/19	-	-	-	-	-	-
Fluopyram (mg kg^{-1})	QA	Feb/18	0.0093	0.0013	13.6	0.009	0.008	0.011
		Apr/18	0.0063	0.00058	9.12	0.006	0.006	0.007
		Jun/18	0.007	-	-	0.007	0.007	0.007
		Jan/19	0.005	-	-	0.005	0.005	0.005
	QG	Feb/18	0.0146	0.0065	44.3	0.016	0.008	0.024
		Apr/18	0.0134	0.0056	42.1	0.012	0.006	0.020
		Jun/18	0.0086	0.0022	25.3	0.009	0.006	0.011
		Jan/19	0.008	0.0036	44.5	0.007	0.005	0.013

Table A10. (Continuation) Descriptive statistics of the concentration of the detected plant protection products in the four sampling periods for both farms studied.

PPP	Farm	Sampling period	Mean	STD	CV (%)	Median	Min	Max
Indoxacarb (mg kg^{-1})	QA	Feb/18	-	-	-	-	-	-
		Apr/18	-	-	-	-	-	-
		Jun/18	-	-	-	-	-	-
		Jan/19	-	-	-	-	-	-
	QG	Feb/18	0.015	0.0083	54.9	0.012	0.006	0.025
		Apr/18	0.016	0.0029	18.4	0.016	0.013	0.019
		Jun/18	0.0095	0.0035	37.2	0.0095	0.007	0.012
		Jan/19	0.011	0.0026	23.5	0.011	0.008	0.014
Iprovalicarb (mg kg^{-1})	QA	Feb/18	-	-	-	-	-	-
		Apr/18	0.012	-	-	0.012	0.012	0.012
		Jun/18	0.041	0.038	938	0.028	0.008	0.11
		Jan/19	-	-	-	-	-	-
	QG	Feb/18	-	-	-	-	-	-
		Apr/18	-	-	-	-	-	-
		Jun/18	-	-	-	-	-	-
		Jan/19	-	-	-	-	-	-
Kresoxim-methyl (mg kg^{-1})	QA	Feb/18	-	-	-	-	-	-
		Apr/18	-	-	-	-	-	-
		Jun/18	0.008	-	-	0.008	0.008	0.008
		Jan/19	-	-	-	-	-	-
	QG	Feb/18	-	-	-	-	-	-
		Apr/18	-	-	-	-	-	-
		Jun/18	-	-	-	-	-	-
		Jan/19	-	-	-	-	-	-
Metalaxyl (mg kg^{-1})	QA	Feb/18	-	-	-	-	-	-
		Apr/18	-	-	-	-	-	-
		Jun/18	-	-	-	-	-	-
		Jan/19	-	-	-	-	-	-
	QG	Feb/18	0.0318	0.0116	36.5	0.027	0.024	0.052
		Apr/18	0.0136	0.00518	38.2	0.011	0.0078	0.020
		Jun/18	0.149	0.164	110	0.071	0.060	0.44
		Jan/19	0.016	0.0034	21.2	0.017	0.012	0.020

Table A10. (Continuation) Descriptive statistics of the concentration of the detected plant protection products in the four sampling periods for both farms studied.

PPP	Farm	Sampling period	Mean	STD	CV (%)	Median	Min	Max
Metrafenone (mg kg^{-1})	QA	Feb/18	-	-	-	-	-	-
		Apr/18	-	-	-	-	-	-
		Jun/18	-	-	-	-	-	-
		Jan/19	-	-	-	-	-	-
	QG	Feb/18	0.0133	0.0036	27.1	0.0145	0.008	0.016
		Apr/18	0.0082	0.00295	36.0	0.0070	0.006	0.013
		Jun/18	0.007	0.0014	20.2	0.0070	0.006	0.008
		Jan/19	0.007	-	-	0.007	0.007	0.007
Oxyfluorfen (mg kg^{-1})	QA	Feb/18	1.233	1.021	82.8	1.095	0.23	3.00
		Apr/18	0.628	0.349	55.6	0.575	0.32	1.30
		Jun/18	0.189	0.138	72.9	0.150	0.035	0.39
		Jan/19	0.089	0.066	74.6	0.093	0.020	0.18
	QG	Feb/18	0.484	0.291	60.1	0.360	0.300	1.00
		Apr/18	0.390	0.157	40.2	0.330	0.230	0.640
		Jun/18	0.162	0.0576	35.6	0.150	0.100	0.240
		Jan/19	0.0488	0.0212	43.6	0.0465	0.027	0.075
Penconazole (mg kg^{-1})	QA	Feb/18	0.0113	0.0039	34.7	0.011	0.007	0.017
		Apr/18	0.007	0.001	14.3	0.007	0.006	0.008
		Jun/18	0.0108	0.0036	33.4	0.012	0.006	0.014
		Jan/19	0.008	0.0032	39.5	0.0075	0.005	0.012
	QG	Feb/18	0.0106	0.0042	39.2	0.011	0.006	0.017
		Apr/18	0.0091	0.00198	21.8	0.009	0.0064	0.011
		Jun/18	0.007	0.0014	20.2	0.007	0.006	0.008
		Jan/19	0.0070	0.0027	38.40	0.0061	0.005	0.011
Phthalimide (mg kg^{-1})	QA	Feb/18	-	-	-	-	-	-
		Apr/18	-	-	-	-	-	-
		Jun/18	0.016	0.001	4.56	0.016	0.015	0.016
		Jan/19	-	-	-	-	-	-
	QG	Feb/18	-	-	-	-	-	-
		Apr/18	-	-	-	-	-	-
		Jun/18	0.009	-	-	0.009	0.009	0.009
		Jan/19	-	-	-	-	-	-

Table A10. (Continuation) Descriptive statistics of the concentration of the detected plant protection products in the four sampling periods for both farms studied.

PPP	Farm	Sampling period	Mean	STD	CV (%)	Median	Min	Max
Pyrethrin (mg kg^{-1})	QA	Feb/18	0.015	-	-	0.015	0.015	0.015
		Apr/18	0.013	-	-	0.013	0.013	0.013
		Jun/18	0.053	-	-	0.053	0.053	0.053
		Jan/19	0.023	0.023	104	0.023	0.006	0.039
	QG	Feb/18	-	-	-	-	-	-
		Apr/18	-	-	-	-	-	-
		Jun/18	-	-	-	-	-	-
		Jan/19	-	-	-	-	-	-
Spiroxamine (mg kg^{-1})	QA	Feb/18	0.018	0.0061	34.3	0.019	0.008	0.024
		Apr/18	0.024	0.025	106	0.0135	0.009	0.074
		Jun/18	0.076	0.041	54.1	0.074	0.032	0.14
		Jan/19	0.20	0.01	51.2	0.020	0.008	0.035
	QG	Feb/18	0.016	0.008	47.4	0.015	0.006	0.027
		Apr/18	0.013	0.005	36.1	0.014	0.006	0.019
		Jun/18	0.117	0.081	69.6	0.091	0.058	0.26
		Jan/19	0.030	0.012	37.8	0.034	0.019	0.046
Tebuconazole (mg kg^{-1})	QA	Feb/18	0.0055	0.0007	12.9	0.0055	0.005	0.006
		Apr/18	-	-	-	-	-	-
		Jun/18	-	-	-	-	-	-
		Jan/19	0.0073	0.0015	20.8	0.007	0.006	0.009
	QG	Feb/18	0.011	0.0041	38.4	0.01	0.006	0.016
		Apr/18	0.0093	0.0023	24.7	0.008	0.008	0.012
		Jun/18	0.0070	0.0026	37.8	0.006	0.005	0.01
		Jan/19	0.0075	0.0017	23.1	0.008	0.0053	0.009
2,4-D (mg kg^{-1})	QA	Feb/18	0.008	0.002	23.4	0.008	0.006	0.011
		Apr/18	-	-	-	-	-	-
		Jun/18	0.008	-	-	0.008	0.008	0.008
		Jan/19	-	-	-	-	-	-
	QG	Feb/18	-	-	-	-	-	-
		Apr/18	-	-	-	-	-	-
		Jun/18	-	-	-	-	-	-
		Jan/19	-	-	-	-	-	-