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Pyrethroid pesticides in soils: assessment and environmental impact

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Tese de Doutoramento apresentada à
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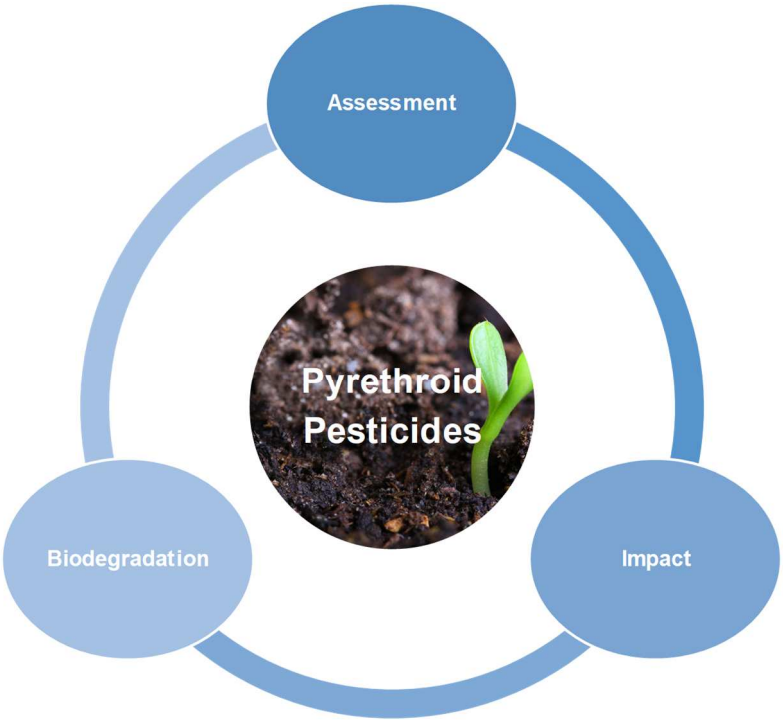

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Doutoramento em Química Sustentável
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Pyrethroid pesticides in soils: assessment and environmental impact

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"*The future belongs to those who believe in the beauty of their dreams*."
Eleanor Roosevelt

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To my baby boy,

"From once just a thought in faraway dreams,
now into my arms and in my eyes gleam
the presence of you.
Your laughter and smiles,
which go on for miles,
warm my heart and soul."

Amy R. Campbell

“Science is a way of life.
Science is a perspective.
Science is the process that takes us from confusion to understanding in a manner that's precise, predictive and reliable - a transformation, for those lucky enough to experience it, that is empowering and emotional.”

Brian Greene

Abstract

Pyrethroid pesticides are widely used as insecticides for controlling insect pests and their intensive use raise environmental concerns. Limited information can be found in the literature regarding contamination of soils by pyrethroid pesticides in playground parks and agricultural areas, a greater knowledge about its impact is needed.

A QuEChERS (Quick Easy Cheap Effective Rugged and Safe) method with gas chromatography (GC) with electron-capture detector (ECD) was implemented to assess pyrethroid pesticides contamination on soils. In this work, new findings related to the spatial and seasonal occurrence of pyrethroid pesticides and their metabolite contaminants in soil environment in Portugal were accomplished. Pyrethroid contamination in measurable levels was not found in the ten playgrounds soil samples analyzed in both seasons (summer and winter). For the eighteen agricultural soil samples tested, deltamethrin was the only pyrethroid detected, just in the summer season. Three out of them were found to be positive in concentration between 15.7 and 101.7 ng g^{-1}. Afterward, an analytical method that combines an aqueous solid-liquid extraction, the solid phase extraction (SPE) and gas chromatography/mass spectrometry (GC/MS) detection have been developed and validated for the determination of 3-phenoxybenzoic acid (3-PBA) in soil samples. This new methodology allows limits of detection and quantification equal to 4.0 and 13.3 ng g^{-1}, respectively. Under optimized conditions the method average recoveries levels ranged from 70.3 and 93.5%. The developed method was applied to eleven agricultural soil samples in the north of Portugal and it allowed the determination of the pyrethroid metabolite, 3-PBA, in one of the eleven agricultural soil samples tested at a level of few ng g^{-1}.

The monitoring over time of a pyrethroid pesticide, deltamethrin, degradation was carried out following their evolution on the applied cabbage and soils. Its progression was correlated with the changes in the natural microbial consortium present, that potentially biodegrade deltamethrin and its metabolite (3-PBA). Shifts in soil microbial community structure were observed after 30 days of pesticide application. The main changes were the increased abundance of *Nocardioides* sp*.* and *Sphingomonas* sp*.,* correlated with deltamethrin and 3-PBA consumption, respectively. Although deltamethrin was not found in any of the tested samples (soil and cabbage) after 180 days, it caused an environmental impact much further than the 7 days of security interval described in this insecticide product flyer.

The phytotoxic impact of selected pyrethroid pesticides and its metabolite 3-PBA was evaluated in *Cucumis sativus* (cucumber) seeds. Percentage of seed germination, root elongation, shoot length and leaf length were considered as endpoints to assess the possible acute phytotoxicity of soil by the exposure to pyrethroid pesticides (cypermethrin, deltamethrin, and cyhalothrin) and 3-PBA, in a concentration range between 50 and 500 ng g^{-1}. A negative impact was observed in germination when seeds were exposed to the metabolite. Cypermethrin showed impact in the three studied endpoints of seed development, while deltamethrin only affected the root length. Concerning pigments content, the median values of chlorophylls and total carotenoids increased for cypermethrin and deltamethrin. This increase was more pronounced to deltamethrin.

These results denote the need for monitoring and assessment of pyrethroid pesticides contamination in soils. Further investigation is required to fully understand the potential ecological impact of pyrethroid pesticides in soils.

Keywords:

Pyrethroid pesticides; 3-Phenoxybenzoic acid; Soils; Contamination; Microbial community; Biodegradation; Phytotoxicity; Seedling growth tests

Resumo

Os pesticidas piretróides são amplamente utilizados como inseticidas no controle de pragas de insetos, todavia o seu uso intensivo gera preocupações ambientais. A informação disponível na literatura sobre a contaminação de solos por pesticidas piretróides em parques infantis e áreas agrícolas é limitada, sendo necessário um maior conhecimento acerca do seu impacto.

O método de extração "QuEChERS (Quick Easy Cheap Effective Rugged and Safe)" seguido de quantificação por cromatografia gasosa (GC) com detetor de captura de eletrões (ECD) foi implementado de forma a avaliar a contaminação dos solos por pesticidas piretróides. Neste trabalho foram obtidos novos conhecimentos relativos à ocorrência espacial e sazonal dos pesticidas piretróides e do seu principal metabolito nos solos do norte de Portugal. Não foram encontrados níveis mensuráveis nas dez amostras de solo de parques infantis analisadas em ambas as estações do ano (verão e inverno). Contudo, para as dezoito amostras de solos agrícolas testadas, a deltametrina foi o único piretróide detetado e apenas na estação de verão. Dos três solos positivos à contaminação por deltametrina, estes apresentaram concentrações entre 15,7 e 101,7 ng g^{-1}. Posteriormente, um método analítico que combina extração sólido-líquido em fase aquosa, uma extração em fase sólida (SPE) e deteção por cromatografia gasosa/espectrometria de massas (GC/MS) foi desenvolvido e validado para a determinação do ácido 3-fenoxibenzóico (3-PBA) em amostras de solos. Esta nova metodologia permitiu limites de deteção e quantificação iguais a 4,0 e 13,3 ng g^{-1}, respetivamente. Sob condições otimizadas, os níveis médios dos índices de recuperação do método variaram de 70,3 e 93,5%. O método desenvolvido foi aplicado a onze amostras de solos agrícolas do norte de Portugal e permitiu a determinação deste metabolito, 3-PBA, numa das onze amostras de solos agrícolas testadas a um nível de poucos ng g^{-1}.

A monitorização ao longo do tempo da degradação de um pesticida piretróide, deltametrina, foi realizada após esta ter sido aplicada num solo plantado com couves. A progressão ao longo do tempo foi correlacionada com as alterações no consórcio microbiano natural presente no solo e que potencialmente degradam a deltametrina e o seu metabolito (3-PBA). Foram observadas mudanças na estrutura da comunidade microbiana do solo após 30 dias da aplicação do pesticida. As principais alterações observadas foram o aumento da abundância de *Nocardioides* sp. e *Sphingomonas* sp., bactérias as quais podem estar relacionadas com o desaparecimento da deltametrina e do 3-PBA, respetivamente. Embora a deltametrina não tenha sido encontrada em nenhuma das amostras testadas (solo e couve) após 180 dias, verificamos que este

composto causou um impacto ambiental muito maior do que o intervalo de segurança de 7 dias descrito na bula do fitofarmacêutico.

O impacto fitotóxico dos pesticidas piretróides (cipermetrina, deltametrina e cialotrina) e do seu metabolito 3-PBA, foi avaliado em sementes de *Cucumis sativus* (pepino) numa gama de concentrações entre 50 e 500 ng g^{-1}. A percentagem de germinação das sementes, o alongamento da raiz, o comprimento do caule, o tamanho das folhas e o seu conteúdo em pigmentos foram os parâmetros utilizados para avaliar a possível fototoxicidade aguda no solo pela exposição aos pesticidas piretróides. Quando as sementes foram expostas ao metabolito foi observado um impacto negativo na sua germinação. A cipermetrina teve influência nos três parâmetros obsevados de desenvolvimento das sementes, já a deltametrina afetou apenas o comprimento das raízes. Quanto ao teor de pigmentos, os valores médios de clorofila e carotenoides totais aumentaram para a cipermetrina e deltametrina. Este aumento foi mais pronunciado para a deltametrina.

Esses estudos denotam a exigência de monitorizar e avaliar a contaminação dos solos por pesticidas piretróides. Existe assim a necessidade de obter mais conhecimento com vista a melhor entender o potencial impacto ecológico dos pesticidas piretróides nos solos.

Palavras-chave:

Pesticidas piretróides; Ácido 3-fenoxibenzóico; Solos; Contaminação; Comunidade microbiana; Biodegradação; Fototoxicidade; Germinação

List of publications

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Publication in international peer-review journals

Idalina Bragança, Ana Paula Mucha, Maria Paola Tomasino, Filipa Santos, Paulo C. Lemos, Cristina Delerue-Matos and Valentina F. Domingues, “Deltamethrin impact in a cabbage planted soil: degradation and effect on microbial community structure”. Submitted.

Idalina Bragança, Paulo C. Lemos, Cristina Delerue-Matos, and Valentina F. Domingues, "Assessment of pyrethroid pesticides in topsoils of northern Portugal ". Submitted.

Idalina Bragança, Paulo C. Lemos, Cristina Delerue-Matos, and Valentina F. Domingues, "Pyrethroid pesticide metabolite, 3-PBA, in soils: method development and application to real agricultural soils ". Submitted.

Idalina Bragança, Paulo C. Lemos, Piedade Barros, Cristina Delerue-Matos, and Valentina F. Domingues (2018), "Phytotoxicity of pyrethroid pesticides and its metabolite towards *Cucumis sativus*," Science of the Total Environment, 619–620, 685-691. https://doi.org/10.1016/j.scitotenv.2017.11.164

Book chapters

Idalina Bragança, Clara Grosso, Diana Rede, Susana R. Sousa, Paulo C. Lemos, Valentina F. Domingues, Cristina Delerue-Matos. “Ecotoxicological effects of insecticides in plants assessed by germination and other phytotoxicity tools”, in S. Vats (ed.) Biotic and Abiotic Stress Tolerance in Plants, Springer Nature Singapore Pte Ltd., 2018, p. 47-76, https://doi.org/10.1007/978-981-10-9029-5_3

Idalina Bragança, Valentina F. Domingues, Paulo C. Lemos, Cristina Delerue-Matos, “Biodegradation of Pyrethroid Pesticides” in: Jose T.V.S. Albergaria, Henri P.A. Nouws (eds.), Soil Remediation: Applications and New Technologies, Boca Raton, USA: CRC Press, Taylor & Francis Group, 2016, p. 59-74, ISBN: 9781498743624

International Communications in Scientific Meetings

Poster contributions

Idalina Bragança, Paulo C. Lemos, P. Barros, Cristina Delerue-Matos and Valentina F. Domingues, Pyrethroid pesticides and its metabolite phytotoxicity evaluation towards cucumber, 40th Environmental & Food Monitoring Conference, Santiago de Compostela, Spain,19-22 June 2018.

Idalina Bragança, Paulo C. Lemos, Piedade Barros, Cristina Delerue-Matos and Valentina F. Domingues, Development and validation of a SPE GC-MS method for the quantification of 3-PBA in soils, 40th Environmental & Food Monitoring Conference, Santiago de Compostela, Spain,19-22 June 2018.

Idalina Bragança, Paulo C. Lemos, Cristina Delerue-Matos and Valentina F. Domingues, Pyrethroid Pesticides on soil: assessment and toxicity, P117, 15th Symposium on Chemistry and Fate of Modern Pesticides, Santiago de Compostela, Spain, 4-7 October 2016.

National Communications in Scientific Meetings

Poster contributions

Idalina Bragança, Paulo C. Lemos, Cristina Delerue-Matos and Valentina F. Domingues Impact of Pyrethroid pesticides use in soils P16, 1st Scientific Meeting of the Doctoral Programme in Sustainable Chemistry, Aveiro, Portugal, 26th September 2016.

Idalina Bragança, Valentina F. Domingues, Paulo C. Lemos and Cristina Delerue-Matos, Assessment of pyrethroid pesticides contaminated soil in Porto city playgrounds, P.018, 9th National Meeting on chromatography, Lisboa, Portugal, 5-9 January 2016.

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List of abbreviations

3-PBAld	3-phenoxybenzaldehyde
3-PBA or 3-PBAcid	3-phenoxybenzoic acid
ABA	Abscisic acid
ACN	Acetonitrile
AOAC	Association of Official Agricultural Chemists
APX	Ascorbate peroxidase
ATP	Adenosine triphosphate
BHC	Benzene hexachloride
C18	Octadecyl
Ch a	Chlorophyll-a
Ch b	Chlorophyll-b
Cha/b	Chlorophyll a/b ratio
Chl	Chlorophyll
Co	Coenzyme
CP	Cypermethrin
Cx+c	Total carotenoids
DDD	Dichloro-diphenyl-dichloroethane
DDE	Dichloro-diphenyl-dichloroethylene
DDT	Dichloro-diphenyl-trichloroethane
DDTs	DDD+ DDE+ DDT
DGAV	Ministry of Agriculture Forestry and Rural Development
DIC	N,N'-Diisopropylcarbodiimide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
dSPE	Dispersive solid-phase extraction
EC50	Dose effect causing an influence of 50% (one half) on the group of test animals
ECD	Electron-capture detector
EI	Electron impact ionization
ELISA	Enzyme-linked immunosorbent assay
EN 15662	EN 15662 QuEChERS method (citrate version)
EPA	Environmental Protection Agency
EU	European Union
FCCs	Fluorescent chlorophyll catabolite

FEN	Fenvalerate
GA	Gibberellins
GC	Gas chromatography
GDH	Glutamate dehydrogenase
GOGAT	Glutamate synthase
GR	Glutathione reductase
GS	Glutamine synthetase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
h	Hours
HCB	Benzene hexachloride
HCH	Hexachlorocyclohexane
HFIP	1,1,1,3,3,3-Hexafluoro-2-propanol
JA	Jasmonic acid
HPLC	High performance liquid chromatography
ISTA	International Seed Testing Association
LC	Liquid chromatography
LC_{50}	Lethal dose which causes the death of 50% (one half) of a group of test animals
LLE	Liquid-liquid extraction
LOD/LODs	Limit of detection/limits of detection
Log P	octanol-water partition coefficient
LOQ	Limit of quantification
M	Molar
mM	Millimolar
ME	Matrix effect
$MgSO_4$	Magnesium sulfate
min	Minute
Min	Minimum value
MS	Mass spectrometry
MS/MS or MS^2	Tandem mass spectrometry
m/z ratio	Mass-to-charge ratio
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NCCs	Nonfluorescent chlorophyll catabolites
OCPs	Organochlorine pesticides
OC	Organic carbon content

OPDA	12-oxo-phytodienoic acid
OCDE	Organization for Economic Co-operation and Development
OPYs	Pyrethroid pesticides
OPs	Organophosphates pesticides
p.a.	pro-analysis puriss
PAL	Phenylalanine ammonia lyase
PCBs	Polychlorinated biphenyls
PCR	Polymerase chain reaction
pka	Negative logarithm of the ionization constant of an acid, a measure of the strength of an acid
PSA	Primary secondary amine
QuEChERS	Quick Easy Cheap Effective Rugged and Safe
R^2	Coefficient of determination
RCC	Red chlorophyll catabolite
ROS	Reactive oxygen species
RSD	Relative Standard Deviation
s	seconds
SA	Salicylic acid
SD	Standard deviation
SPE	Solid phase extraction
SIM	Selected-ion monitoring
SOD	Superoxide dismutase
SPs	Synthetic pyrethroids
S/N	Signal-to-noise ratio
t	Time
$t_{1/2}$ or $T_{1/2}$	Half-live time
TOC	Total organic carbon
ToF	Time-of-flight
US	United States
USEPA	United States Environmental Protection Agency
UV	Ultraviolet
WHO	World Health Organization

PREFACE

This section presents the relevance and motivation, the main objectives and organization, and structure of the thesis.

Preface

Relevance and motivation

Agricultural external inputs such as pesticides, organic amendments, and fertilizers are applied to maximize productivity. Pesticides, in general, allowed a remarkable increase in agricultural yield and food production (Aktar et al. 2009). This is an important factor to worldwide agricultural systems as they enable that agricultural production follows the growing trend of world population increase (Carvalho 2017). Therefore, pesticides are being used all over the world, whereas side effects on soil microorganisms are frequently neglected. Synthetic pyrethroids (SPs) are one of the most used pesticides classes (Burns and Pastoor 2018), and its utilization greatly increased in the last decades due to their effectiveness and low toxicity when compared to other insecticides (Yoo et al. 2016). Their constant application can cause effects to human health and to the environment (life forms and the ecosystem) as they became persistent in the environment (Tang et al. 2018). Nature can many times solve these problems by itself, finding bacteria and fungi able to perform the task of biodegradation. There is still a lack of knowledge about these insecticides' effects to microorganisms in the contaminated areas and their impact in soils ecosystem.

Objectives

Concerning the increasing use of pyrethroid pesticides the present work intended as major goals the assessment of pyrethroid pesticides soil contamination and their environmental impact. For this purpose, different specific objectives were established:

- development and/or validation of efficient extraction techniques and analytical methodologies for determination of pyrethroids and their major metabolite in soils;
- evaluate the most frequents pyrethroids in Portuguese soils;
- evaluate the bioremediation of the contaminated soil with the local microbial population;
- investigate the potential impact of pyrethroids' application.

Organization and structure of the thesis

The present thesis includes all the work developed under the scope of this doctoral project. It was divided into five chapters that enclosed a compilation of the book chapters and scientific articles produced in these four years. For all the book chapters and articles, the original structures were maintained in agreement with where they were published or submitted. Firstly, the relevance, main objectives, and thesis outline were provided here in this Preface chapter.

The contextualization of the developed work was given in Chapter 1, with a theoretical background. This chapter first summarized a general overview (Section 1.1) and then published data in two main topics (Section 1.2 and 1.3). For Section 1.2 the biodegradation of pyrethroid pesticides was considered, with an overview of pyrethroids structure, mode of action and impact, microorganism's strains (bacteria and fungi), and enzymes that hold the potential to be used in the bioremediation of pyrethroid contaminated soils. Section 1.3 included a review of the topics related with ecotoxicological aspects of insecticides present in the soil environment, as soils contamination by insecticides, tests to access phytotoxicity and germination tests as a tool to access phytotoxicity by insecticides.

The experimental work developed during this doctoral project was divided into three chapters (Chapter 2 to 4). Chapter 2 assessed the pyrethroid pesticides soil contamination. Section 2.1 was focused in the spatial and seasonal occurrence of pyrethroid pesticides contaminants in soil environment in Portugal. Two types of soils (ten playgrounds and eighteen agricultural soil samples) were evaluated for pyrethroids presence in two seasons (summer and winter) in the north of Portugal. A greener analytical technique, a QuEChERS (Quick Easy Cheap Effective Rugged and Safe) method with gas chromatography (GC) with an electron-capture detector (ECD), was used to achieve this goal. In order to extend the study of pyrethroids in the environment to their metabolites, in Section 2.2 the work also included pyrethroids principal metabolite (3-phenoxybenzoic acid) determination. This work allowed, for the first time, the development and validation of 3-PBA analytical determination and its application to real soil samples. This analytical method combined an aqueous solid-liquid extraction, a solid phase extraction (SPE) and gas chromatography/mass spectrometry (GC/MS) detection. In Chapter 3 and Chapter 4 two different applications in the environmental field to assess pyrethroids impact were presented. In the former (Chapter 3), monitoring of the deltamethrin pyrethroid pesticide degradation over time was carried out by following their evolution on the applied cabbage and soils. Its progression was correlated with the changes in the natural microbial consortium present, that could

potentially biodegrade deltamethrin and its metabolite, 3-PBA. In Chapter 4, the phytotoxicity posed by some pyrethroid pesticides (cypermethrin, deltamethrin, and cyhalothrin) and its metabolite 3-PBA was also evaluated in *Cucumis sativus* (*C. sativus*) seeds. Percentage of seed germination, root elongation, shoot length, and leaf length were considered as endpoints to assess the possible acute phytotoxicity of soil. Furthermore, the pigments content of leaves (chlorophylls and carotenoids content) were also of the main parameters followed.

Finally, in Chapter 5 the final concluding remarks were presented. Here, the main conclusions of the developed work were highlighted as well pointed out the future perspectives.

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CHAPTER 1

Introduction

1.1

Pyrethroid pesticides: a general overview

1.2

Biodegradation of pyrethroid pesticides

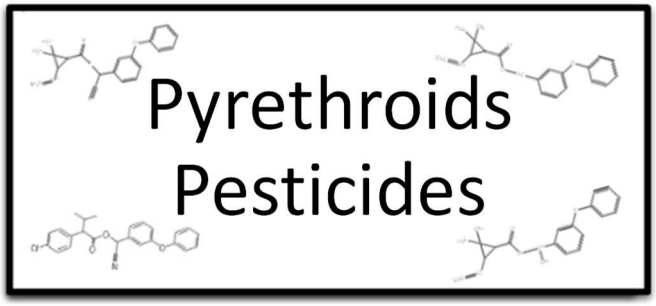
1.3

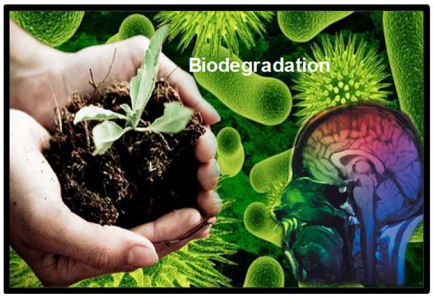
Ecotoxicological effects of insecticides in plants assessed by germination and other phytotoxicity tools

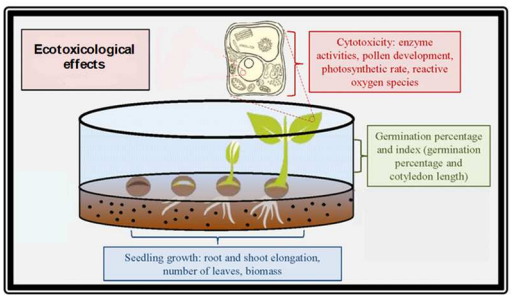
This 1st chapter presents an overview of pyrethroid pesticides and metabolite in soils: occurrence, biodegradation and phytotoxicity.

1.1 Pyrethroid pesticides: a general overview

Tanacetum flowers







Pyrethroid pesticides: a general overview

Synthetic pyrethroid pesticides (SPs) are pesticides chemical derived from pyrethrins (natural compounds extracted from *Tanacetum* flowers) that are widely used as insecticides in agriculture, domestic applications and veterinary (Ensley 2012). The use of these pesticides has grown in recent years because they have advantages compared to other pesticides, such as their selectivity, easy degradation in the environment and low acute toxicity to mammals (Albaseer et al. 2011; Bronshtein et al. 2012; Yoo et al. 2016). Besides, their increasing application raise concerns about its effects on several environmental compartments (Tang et al. 2018), non-target organisms such as aquatic invertebrates (Wang et al. 2011), and its long-term effects (chronic toxicity) on animals and humans (Albaseer et al. 2011). Acute toxicity for SPs occupationally exposed is unlike to occur if good work practices and safety precautions were followed. Nevertheless, 573 cases of acute occupational pyrethroid poisoning resulting from inappropriate handling were reported firstly in China between 1983-1988. Most of the cases involved exposure to deltamethrin (325 cases), followed by fenvalerate (196 cases), cypermethrin (45) and other pyrethroids (7) (He et al. 1989). Deltamethrin is an α-cyano pyrethroid insecticide highly used in pest control. Although was not initially given great importance to its toxicity, studies have emerged giving a special focus to deltamethrin in reviews on pyrethroid toxicity (Rehman et al. 2014). There are studies that show that SPs have carcinogenic potential and can produce metabolites with endocrine activity (Xie et al. 2008). In addition, studies in rats have suggested that SPs may integrate the contribution of the environmental component in the etiology inducing Parkinson's disease (Nasuti et al. 2007; Weiner et al. 2009).

Several studies have reported a global occurrence of pyrethroid (Tang et al. 2018) but there is still a lack of information on levels of soil and sediment contamination in Europe (Pistocchi et al. 2009), the main monitoring studies of these compounds have been carried out in China (Liu et al. 2016; Yao et al. 2010), United States (Riederer et al. 2010) and India (Kumari et al. 2008; Murugan et al. 2013). However, there are some studies that point to contamination levels in river water and sediments in Spain. Feo et al. (2010) found the pyrethroids cypermethrin and deltamethrin in Ebro River Delta water in levels between 0.73 ng L^{-1} to 57.2 ng L^{-1} and 2 ng L^{-1} to 58.8 ng L^{-1} for cypermethrin and deltamethrin, respectively. Only cypermethrin, in a total of 12 pyrethroids analyzed, were detected in the sediments in ranging levels between 8.27 ng g^{-1} to 71.9 ng g^{-1}.

To evaluate SPs in soil samples several extraction techniques such as: ultrasound-assisted extraction (Shi et al. 2011), microwave assisted extraction (Esteve-Turrillas et al. 2004; Zhang et al. 2012), headspace solid-phase microextraction (Fernandez-Alvarez et al. 2008) and QuEChERS (quick, easy, cheap, effective, rugged, and safe) method (Yang et al. 2010), are used followed by gas chromatographic analysis.

QuEChERS method is a greener analytical technique, has it has numerous advantages over most of the traditional extraction techniques such as low solvent consumption and high efficiency with a low number of steps (Vera et al. 2014).

The problem of environmental contamination by SPs should be properly evaluated and the use of remediation techniques for efficient decontamination of soils is also necessary and urgent. The application of bioremediation techniques is an inexpensive process, which uses the natural mechanisms of soil degradation, through the specific microorganisms present therein or, if necessary, enrichment with these or other microorganisms (Soares et al. 2010). There are several reported bacteria (*Acidomonas* sp., *Bacillus* sp., *Pseudomonas* sp. and *Sphingomonas* sp.) and fungi (*Aspergillus* sp., *Trichoderma* sp. and *Cladosporium* sp.) capable of degrading SPs and its metabolite in contaminated environmental samples. Insecticides breakdown by microorganisms has been considered the most important catabolic reaction in soil and this process can be crucial for pyrethroid-contaminated soils decontamination (Bragança et al. 2016).

The use of pyrethroid insecticides cannot only cause residues in the soil but even leads to detrimental effects on host-plant tissues plants, which necessitates a thorough understanding of their phytotoxicity. Research on the effects of pyrethroids on seed germination are scarce (Hanley and Whiting 2005; Moore and Locke 2012). Subsequently, a phytotoxic impact of these pesticides on soil is also need to access the plant damage due to their application. Pesticide phytotoxicity appears in several ways on plants, causing an impact on plants. Plants are at their most sensitiveness to chemical's application during the early stages of life (Hewitt and Rennie 1986). As plants are sensitive to harmful substances they can be used as bioindicators in toxicity assessment studies (Kapanen and Itävaara 2001) as these assays are direct, inexpensive, and gives an integrated estimation of bioavailability and contaminant toxicity (Maila and Cloete 2005). Inhibition of germination or on the root, shoot, and leaf development are the main areas of interest in studies on phytotoxicity (Kapanen and Itävaara 2001). Moreover, insecticides sprayed over the leaves can also affect photosynthetic efficiency by increasing or decreasing pigment contents and affecting the electron transport chain in chloroplasts (Bashir et al. 2014; Chopade et al. 2007).

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1.2 Biodegradation of pyrethroid pesticides

Soil Remediation: Applications and New Technologies, Boca Raton, USA: CRC Press, Taylor & Francis Group (2016) p. 59-74, ISBN: 978-1-4987-4361-7

Statement of contribution

The contribution of the candidate, Idalina Bragança, in this work includes the literature review about pyrethroids structure, mode of action and impact, and biodegradation by bacteria, fungi and enzymes. Write of the book chapter regarding to the subjects researched.

CHAPTER 4

Biodegradation of Pyrethroid Pesticides

Idalina Bragança,[1,a] *Valentina F. Domingues*,[1,*] *Paulo C. Lemos*[2] and *Cristina Delerue-Matos*[1,b]

ABSTRACT

Pyrethroids are widely used as insecticides in agriculture, veterinary, and domestic applications. Their increasing use has become an environmental concern. Pyrethroid pesticides (SPs) residues have been frequently detected in soils and thus in agricultural samples. Some authors considered the microbial breakdown of insecticides the most important catabolic reaction in soil and can be crucial for development of pesticide decontamination. The available biodegradation reports showed that the majority of the studies involving the biodegradation of pyrethroid pesticides are done by pure cultures, considering isolated organisms (Bacteria and Fungi) capable of proficiently degrading different SPs even when present as the sole carbon source. Several pyrethroid-hydrolyzing enzymes have consistently been purified and characterized from various resources including metagenomes and organisms. This chapter shows that microorganisms' strains hold potential to be used in bioremediation of pyrethroid contaminated soils.

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Introduction

The use of pesticides is important and essential to protect and facilitate agricultural productivity, since pests are largely responsible for losses incurred during the production of food. Pests can be insects, mice, fungi, microorganisms, and undesirable plants (van der Hoff and van Zoonen 1999). Classification of pesticides can be made according to the Environmental Protection Agency (EPA) regarding the type of pest targeted (e.g., insecticides, fungicides, algaecide, herbicides, and nematicides) but they can also be classified regarding their chemical properties namely organophosphate, organochlorine, carbamate, and pyrethroid pesticides. The World Health Organization (WHO) recommended the classification of pesticides by hazard, that distinguishes between the more and the less hazardous forms of each pesticide, being based on the toxicity of the compound. Pyrethroid pesticides are synthetic insecticides derived from pyrethrins, natural compounds present in the pyrethrum extract from *Tanacetum cinerariae folium* (Kaneko 2010). The main active constituents of the extract are pyrethrin I and pyrethrin II with smaller amounts of the related cinerins and jasmolins. As can be seen in Fig. 1, the structural difference between the pyrethrum extract constituents is that pyrethrin I, cinerin I, and jasmolin I have a monocarboxylic acid (ester of chrysanthemic acid) while pyrethrin II, cinerin II, and jasmolin II have a dicarboxylic acid (ester of pyrethric acid) (Gosselin et al. 1984; Wakeling et al. 2012).

Synthetic pyrethroids were developed to preserve the insecticidal activity of pyrethrins and to enhance physical and chemical properties, such as increased stability in light (Gosselin et al. 1984). Pyrethroids are distinguished by three general characteristics: extreme hydrophobicity, rich stereochemistry, and broad-spectrum high-level insecticidal activity.

Pyrethroids are widely used as insecticides in agriculture, veterinary, and domestic applications (Albaseer et al. 2011; Bronshtein et al. 2012). The wide use of such pesticides in recent years is due to their advantages compared with other pesticides, such as its selectivity, easy degradation in the environment, and low acute toxicity to mammals (Albaseer et al. 2011; Bronshtein et al. 2012).

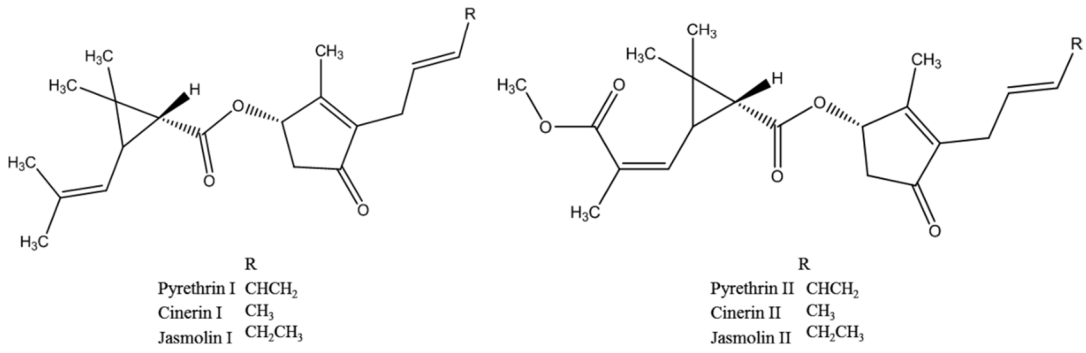

Fig. 1. Molecular structure of the six constituents of pyrethrum extract.

SPs unlike the naturally occurring pyrethrins are stable and persist longer in the environment and capture greater biological activity (Temple and Smith 1996). Pyrethroids may be classified by the presence or absence of a cyano group as type I if they are non-cyanopyrethroids or type II if they have the cyanogroup. Permethrin is an example of a type I pyrethroid while cypermethrin is a type II pyrethroid (Fig. 2) (Mullaley and Taylor 1994; Kurihara et al. 1997).

Worldwide pesticides consumption greatly increased following the increase of population and food production. Due to their extensive use, pyrethroid pesticides residues have been frequently detected in soils, sediments (Weston et al. 2013), and groundwater (Gonçalves et al. 2007).

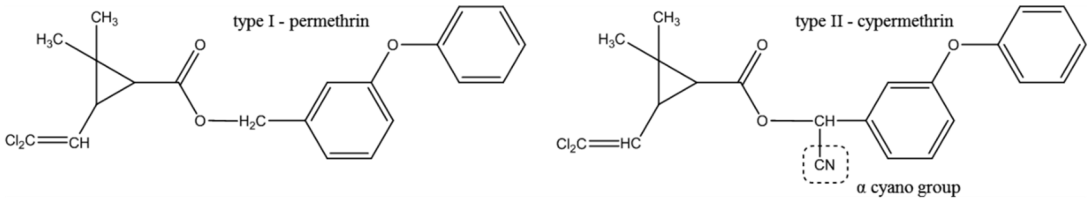

Fig. 2. Molecular structure of type I (permethrin) and type II (cypermethrin) synthetic pyrethroid pesticides.

Pyrethroid detections in environmental samples are commonly performed by gas chromatography (GC) coupled with the electron-capture detector (ECD) (Domingues et al. 2009) or mass spectrometry (MS), although there are other options based on tandem MS (MS^2) or comprehensive two-dimensional GC (GC × GC) coupled with time-of-flight MS (ToF-MS) (Feo et al. 2010). Contamination of agricultural samples (Columé et al. 2001) like fruits (Iñigo-Nuñez et al. 2010), vegetables (Akoto et al. 2013), and tea leaves (Nakamura et al. 1993) may result from preceding treatments in the soil and cross-contamination. The application of pyrethroids for veterinary purpose can be a source of contamination in animal derived products such as milk. Deltamethrin and cypermethrin were detected in residual levels in milk and lactea drink samples, which can be related to their wide use in dairy farming to kill ticks (Goulart et al. 2008).

The use and abuse of pyrethroid pesticides becomes a problem to the environment and thus to human health (Zhang et al. 2011b). Pyrethroids are recognized to contribute to sediment toxicity when found in concentrations which exceed the level expected to cause toxicity. For these reasons, remediation of pyrethroids contaminated soils is paramount to avoid public health hazards. Degradation of pyrethroid insecticides was observed to be more rapid with microorganisms versus sterilized soils, indicating that biological processes do contribute to breakdown in soil. Microbial degradation of SP appears to be a significant breakdown route of such pesticides (Palmquist et al. 2012). Microorganisms, as bacteria and fungi, are known to be primarily responsible for pesticide biodegradation in soil since they have several esterase enzymes capable of degrading pyrethroids (Singh 2002).

Biodegradation of pyrethroid insecticides by bacteria was observed to be more rapid than natural process. The half-lives ($T_{1/2}$) in the presence of the bacteria reported were significantly lower than the ones reported by Laskowski (2002). Pyrethroid pesticides are usually converted to 3-phenoxybenzaldehyde (3-PBAld) and 3-phenoxybenzoic acid (3-PBAcid) (Fig. 3).

In this chapter, some of the current knowledge on several aspects regarding pyrethroid pesticides, such as mode of action and impact on the environment and humans is discussed. An overview of the biodegradation of pyrethroids by different microorganisms is also given.

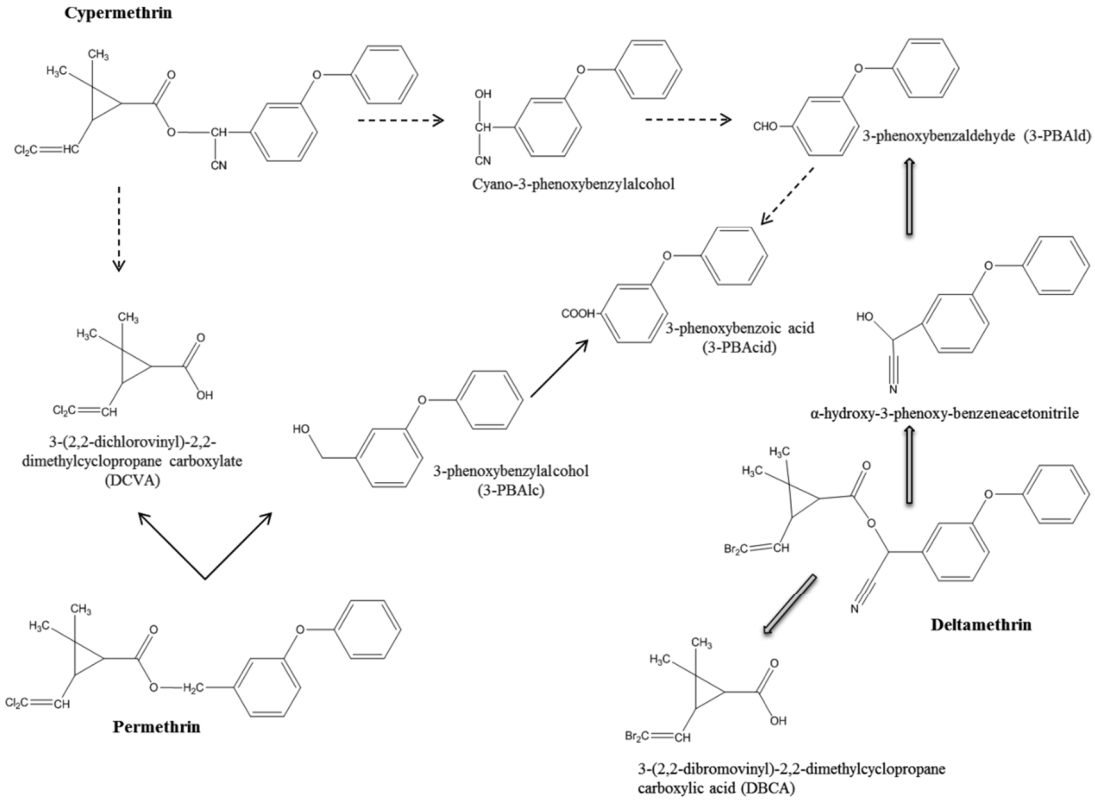

Fig. 3. Degradation pathway of some pyrethroid pesticides (Maloney et al. 1988; Liang et al. 2005; Tallur et al. 2008; Chen et al. 2011c).

Mode of Action and Impact

Pyrethroids are considered neurotoxins since they affect the neuronal function. They act on neuronal receptors and/or ion channels (Zhou et al. 2011). These insecticides act primarily on sodium channels, which are essential to membrane excitability for generation and propagation of nerve action potentials (Dong 2007). However, sensitive calcium and chloride channels can also be an important target of pyrethroid action (Shafer and Meyer 2004). Pyrethroids type I and II share a similar mode of action, as they inhibit insects' nervous systems by keeping the sodium ion channels in nerve

cell membranes open. Type I pyrethroids elicit depolarizing after potentials and repetitive discharges, whereas type II pyrethroids cause stimulus-dependent membrane depolarization leading to conduction block voltage-dependent membrane (Wakeling et al. 2012). Ray and Fry (2006) provided an overview of studies suggesting that the principal effects of pyrethroids were: neurotoxicity development, production of neuronal death, and action mediated via pyrethroid metabolite.

Regarding the persistence of SP in the environment, they became a concern due to their effect on non-target organisms. Although having a relatively low toxicity for mammals and birds, they are extremely toxic for fish and a variety of aquatic invertebrates (Khan 1983). Their increasing application generates concerns about their effects on aquatic invertebrates (Wang et al. 2011), long-term effects, and chronic toxicity in animal species and in humans (Albaseer et al. 2011). Human exposure to these compounds is high, with estimates indicating that 70% of the US population has detectable levels of pyrethroid metabolites in their urine (Ross 2011). There are studies that show that pyrethroid pesticides should be considered as hormone disruptors (Go et al. 1999), have carcinogenic potential (Shukla et al. 2002), and produce metabolites with endocrine activity (Tyler et al. 2000). Furthermore, studies in rats suggested that they can integrate the contribution of the environmental inductive component in the etiology of Parkinson's disease (Nasuti et al. 2007).

Some reports have seen effects of systemic poisoning by SPs. The type I pyrethroids produce tremors and reflex hyperexcitability (T intoxication syndrome). The type II variants produce symptoms that include choreoathetosis, salivation, and seizures (CS intoxication syndrome). Both types potentiate activation of the sympathetic nervous system (Ray and Forshaw 2000; Soderlund et al. 2002). Some pyrethroids clearly exhibit the historic classification symptoms of the T and CS syndromes while others do so less obviously and others, such as fenpropathrin, exhibited features of both groups (Weiner et al. 2009).

Overall, studies imply that each pyrethroid is unique in its ability to influence several cellular pathways. These findings suggest that pyrethroids can be toxic and hormone disruptors and their potential to affect humans and environmental ecosystems should be further investigated.

Biodegradation

Bioremediation is a very promising technology with great potential for contaminated soils (Zouboulis and Moussas 2011). Bioremediation is increasing due to its efficiency and low costs associated compared with other technology (Vidali 2001). This process is mainly based on the utilization of soil microorganisms to degrade or detoxify pollutants. Microorganisms are primarily responsible for pesticide degradation in soil, with the microbial breakdown of insecticides being considered the most important catabolic

reaction taking place in soil. Microorganisms use pesticides present in the environment for normal metabolic processes as carbon or phosphorus sources or other sources of food and energy. This microbial biodegradation process can be crucial when performing pesticide decontamination of the environment (Singh 2002).

To evaluate biodegradation kinetics of the SP some authors used a first order model $C_t = C_0 e^{-kt}$, where k is the rate constant (day^{-1}), C_0 is the amount of compound at time zero, and C_t is the amount of compound at time t (days). The degradation half-life ($T_{1/2}$) is the time in which the compound concentration is reduced by 50%. The $T_{1/2}$ was determined by $T_{1/2} = \ln 2/k$. Concerning the obtained R^2, the pyrethroids biodegradation fit well with this model (Chen et al. 2011a, 2011c, 2011d). Other authors also used a second-order kinetic model to describe the biodegradation (Chen et al. 2012b). Usually, the reported $T_{1/2}$ for SPs in water varies from 17 to 600 days. Pyrethroids are degradable in soils with half-lives ranging from 3 to 96 days aerobically, and 5 to 430 days an aerobically (Laskowski 2002). However, pyrethroid persistence under aerobic soil conditions (field conditions) is highly variable. The half-lives can vary from 11.5 days for cyfluthrin to 96.3 days for bifenthrin. Degradation of pyrethroid insecticides was observed to be more rapid in natural versus sterilized soils, indicating that biological processes do contribute to the breakdown of these contaminants (Palmquist et al. 2012).

Bacteria

Bacteria have a large genetic diversity with the potential to be used as a resource for the development of bioremediation processes. Several studies investigated the biodegradation of pyrethroid pesticides by these microbes in a controlled laboratory environment (Table 1).

The majority of the studies involving the biodegradation of pyrethroid pesticides were done with pure bacterial cultures.

Acidomonas sp. was capable of using allethrin as the sole carbon source and degraded more than 70% of allethrin in minimal medium within three days. The intermediates 2-ethyl-1,3-dimethyl-cyclopent-2-ene-carboxylic acid and cyclopropanecarboxylic acid, 2,2-dimethyl-3-(2-methyl-1-propenyl) were the major metabolites of allethrin biodegradation (Paingankar et al. 2005).

Most studies reporting SPs biodegradation by bacteria were done with cypermethrin and its active stereoisomers. *Azoarcus indigens* HZ5 was able to degrade 90% of β-cypermethrin in a mineral salts medium after six days. The metabolites formed were identified as 3-phenoxybenzaldehyde and 3-phenoxybenzoic acid (Ma et al. 2013). Cypermethrin was also degraded by *Micrococcus* sp. CPN 1, as sole energy source, in mineral salt medium being completely degraded. For the latter organism, cypermethrin was broken down to 3-phenoxybenzoate followed by protocatechuate, phenol, and then complete mineralization was achieved (Tallur et al. 2008). *Pseudomonas*

Table 1. Pyrethroids pesticides degradation by several bacterial species.

Pesticides/ metabolite	Bacteria	Metabolites formed	Degradation	Reference
Allethrin	*Acidomonas* sp.	Major metabolites: Allethrolone; chrysanthemic acid; 2-ethyl-1,3 dimethyl cyclopent-2-enecarboxylic acid; cyclopropanecarboxylic acid and 2,2-dimethyl-3-(2-methyl-1-propenyl)	Allethrin (16 mM) was reduced to 49 and 25% initial concentration after 2 and 3 days of biodegradation, respectively, in minimal medium	(Paingankar et al. 2005)
β-Cypermethrin	*Azoarcusindigens* HZ5	3-phenoxybenzaldehyde and 3-phenoxybenzoic acid	β-cypermethrin in medium was 90% degraded after 6 days	(Ma et al. 2013)
Cypermethrin	*Bacillus* sp. ISTDS2	3-phenoxy phenyl hydroxyacetonitrile, carboxylate, 3-(2, 2-dichloroethenyl)-2, 2-dimethylcyclopropanecarboxylate (DCVA) and 3-PBA	Complete degradation of cypermethrin in soil microcosm after 30 days	(Sundaram et al. 2013)
	Bacillus cereus ZH-3 *Streptomyces aureus* HP-S-01	4-phenoxyphenyl-2,2-dimethyl-propiophenone; 3-phenoxybenzaldehyde and α-hydroxy-3-phenoxy-benzeneacetonitrile	$T_{1/2}$ in medium by *B. cereus* ZH-3: 1.4 days; *S. aureus* HP-S-01: 1.8 days and mixed co-culture: 0.5 days Mixed cultures completely metabolized cypermethrin (50 mg L^{-1}) in medium within 3 days	(Sundaram et al. 2013)
	Micrococcus sp. CPN 1	3-phenoxybenzoate; protocatechuate; phenol and then complete mineralization	Cypermethrin at 1 g L^{-1} in medium as the sole energy source was completely degraded	(Tallur et al. 2008)
Cyfluthrin Cyhalothrin Fenpropathrin Deltamethrin Bifenthrin β-Cypermethrin	*Brevibacteriumaureum* DG-12	Cyfluthrin degradation: 2,2,3,3-Tetramethyl-cyclopropanemethanol; 4-Fluoro-3-phenexy-benzoic acid; 3,5-Dimethoxy phenol and Phenol	$T_{1/2}$ in medium (days): cyfluthrin 1.3; cyhalothrin 1.0; fenpropathrin 1.5; deltamethrin 1.7; bifenthrin 1.9 and β-cypermethrin 1.9. Cyfluthrin, cyhalothrin, fenpropathrin, deltamethrin, bifenthrin, and	(Chen et al. 2013)

Table 1 contd....

Table 1 contd.

Pesticides/ metabolite	Bacteria	Metabolites formed	Degradation	Reference
			β-cypermethrin were degraded 87.4%, 89.1%, 82.6%, 80.9%, 80.1% and 78.3% in medium after 5 days	
β-Cypermethrin 3-PBAcid β-Cyfluthrin Fenpropathrin, Cyhalothrin Deltamethrin	*Ochrobactrum lupin* DG-S-01	------	$T_{1/2}$ in medium (days): β-cypermethrin 1.9; β-cyfluthrin 2.3; fenpropathrin 2.7; cyhalothrin 6.5; deltamethrin 8.2. Degradation kinetics rates in medium within 5 days were: β-cypermethrin 90.4%, β-cyfluthrin 80.8%, fenpropathrin 74.4%, cyhalothrin 56.2%, and deltamethrin 43.0%. 3-PBAcid (25–50 mg L^{-1}) in medium was 90% degraded within 9 days.	(Chen et al. 2011a)
β-Cyfluthrin	*Pseudomonas stutzeri* S1	α-cyano-4-fluoro-3-phenoxybenzyl-3(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate; 4-fluoro-3-phenoxy-a-cyanobenzylalcohol and 3(2,2-dichlorovinyl)-2,2-dimethyl cyclopropanecarboxylic acid	94% degradation of β-cyfluthrin in medium within 8 days	(Saikia et al. 2005)
Fenpropathrin, Cypermethrin Permethrin Cyhalothrin Deltamethrin Fenvalerate Bifenthrin	*Sphingobium* sp. JZ-2	Fenpropathrin degradation: 3-phenoxybenzaldehyde; 3-phenoxybenzoate; protocatechuate and catechol	Complete degradation of fenpropathrin (50 mg L^{-1}) in medium after 4 days incubation. Permethrin and cypermethrin degraded slower than fenpropathrin but faster than fenvalerate, deltamethrin, and cyhalothrin. Bifenthrin (the most persistent) was only 25% degraded within 4 days	(Guo et al. 2009)

Deltamethrin 3-PBAld Cyfluthrin Bifenthrin Fenvalerate Fenpropathrin Permethrin Cypermethrin	*Streptomyces aureus* HP-S-01	Deltamethrin degradation: α-Hydroxy-3-phenoxy-benzeneacetonitrile; 3-Phenoxybenzaldehyde and 2-Hydroxy-4-methoxy benzophenone	Completely removed deltamethrin (50–300 mg L^{-1}) in medium within 7 days. Complete degradation of 3-phenoxybenzaldehyde at 50, 100, and 200 mg L^{-1}, a was observed after 6, 7, and 7 days of incubation,respectively. $T_{1/2}$ in medium (days): cypermethrin 1.57; cyfluthrin 0.87; fenpropathrin 1.32; bifenthrin 0.90; deltamethrin 0.80; fenvalerate 1.08 and permethrin 1.56	(Chen et al. 2011c)
Fenvalerate 3-PBAcid Deltamethrin β-Cypermethrin β-Cyfluthrin Cyhalothrin	*Stenotrophomonas* sp. ZS-S-01	Fenvalerate degradation: 3-PBAcid and then complete mineralization	$T_{1/2}$ in medium (days): Fenvalerate 1.2, deltamethrin 1.3, 3-PBA 1.6, β-cypermethrin 1.9, β-cyfluthrin 2.0 and cyhalothrin 4.0 Fenvalerate was 80% removed from soil within 5 days	(Chen et al. 2011d)
Cis-Bifenthrin Permethrin	*Stenotrophomonas acidaminiphila* (BF6, BF24, BF28); *Aeromonas sóbria* (PM-1); *Erwinia carotovora* (PM-2); *Yersinia frederiksenii* (PM-5)	-------	$T_{1/2}$ in medium (days): cis-bifenthrin: *S. acidaminiphila* BF6-8, *S. acidaminiphila* BF24-58, *S. acidaminiphila* BF28-12 $T_{1/2}$ in medium (days): cis-permethrin: *sobria*-1.4, *E. carotovora*-1.3, *Y. frederiksenii*-1.5	(Liu et al. 2005)
Bifenthrin	*Stenotrophomonas acidaminiphila* (BF6, BF24, BF28)	-------	$T_{1/2}$ (days) in aqueous phase 1.3 to 5.5 $T_{1/2}$ (days) in field sediment 14.3 to 19.4	(Lee et al. 2004)
3-PBAcid	*Sphingomonas* SP-SC-1	Phenol; catechol and 2-phenoxyphen	Completely degraded within 1 day in medium	(Tang et al. 2013)
	Bacillus sp. DG-02	3-(2-methoxyphenoxy) benzoic acid, protocatechuate, phenol, and 3,4-dihydroxy phenol	$T_{1/2}$ in non-sterilized and sterilized soils inoculated with *Bacillus* sp. DG-02: 3.4 and 4.1 days, respectively	(Chen et al. 2012a)

aeruginosa CH7 was not only able to degrade β-cypermethrin but also utilized it as the sole carbon and energy source for growth and biosurfactant production (Zhang et al. 2011a).

Aco-culture of *Bacillus cereus* ZH-3 and *Streptomyces aureus* HP-S-01 significantly enhanced the degradation of cypermethrin. In the mixed co-cultures, lower half-lives ($T_{1/2}$ = 0.5 days) of cypermethrin were observed, as compared to the ones of the pure culture ($T_{1/2}$ = 1.4–1.8 days) (Chen et al. 2012b).

A bacterial strain isolated from active sludge, *Brevibacterium aureum* DG-12, was found effective to degrade cyfluthrin (87.4%), cyhalothrin (89.1%), fenpropathrin (82.6%), deltamethrin (80.9%), bifenthrin (80.1%), and β-cypermethrin (78.3%) in medium after five days. This bacterium degraded cyfluthrin by cleavage of both the carboxylester linkage and diaryl bond to form 2,2,3,3-tetramethyl-cyclopropanemethanol, 4-fluoro-3-phenexy-benzoic acid, 3,5-dimethoxy phenol, and phenol (Chen et al. 2013).

Ochrobactrum lupin DG-S-01 efficiently utilizes β-cypermethrin, β-cyfluthrin, fenpropathrin, cyhalothrin, and deltamethrin as growth substrate. The degradation kinetics rates reached up to 90.4%, 80.8%, 74.4%, 56.2%, and 43.0% within five days, respectively (Chen et al. 2011a).

Pseudomonas stutzeri S1, an isolate from an enrichment culture, was capable of degrading 94% of β-cyfluthrin in mineral salts medium within eight days. Products formed during degradation were identified as α-cyano-4-fluoro-3-phenoxybenzyl-3(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate, 4-fluoro-3-phenoxy-α-cyanobenzylalcohol and 3(2,2-dichlorovinyl)-2,2-dimethyl cyclopropanecarboxylic acid (Saikia et al. 2005).

Sphingobium sp. JZ-2 completely degraded fenpropathrin in mineral salts medium after four days of incubation. Permethrin and cypermethrin were degraded slower than fenpropathrin but faster than fenvalerate, deltamethrin, and cyhalothrin. Only 25% of bifenthrin, the most persistent one, was degraded within four days (Guo et al. 2009).

Deltamethrin in medium was completely removed within seven days with a half-life of 0.80 days by a newly isolated *Streptomyces aureus* HP-S-01. Deltamethrin was metabolized by hydrolysis and produced α-hydroxy-3-phenoxybenzeneacetonitrile and 3-phenoxybenzaldehyde. This strain was also found to be highly efficient in degrading cyfluthrin, bifenthrin, fenvalerate, fenpropathrin, permethrin, and cypermethrin with slightly lower half-lives, ranging from 0.87 to 1.57 days (Chen et al. 2011c).

Fenvalerate was 80% removed from soil within five days by a newly isolated bacterium from activated sludge *Stenotrophomonas* sp. ZS-S-01. Fenvalerate and its hydrolysis product 3-PBAcid were degraded in medium with half-lives from 2.3 to 4.9 days. These bacteria could also degrade and utilize as growth substrates deltamethrin, β-cypermethrin, β-cyfluthrin, and cyhalothrin (Chen et al. 2011d).

Stenotrophomonas acidaminiphila BF6, BF24, and BF28 were capable of degrading bifenthrin with a $T_{1/2}$ between 1.3 to 5.5 days in aqueous phase and 14.3 to 19.4 days in field sediment (Lee et al. 2004). Using cis-bifenthrin

other authors observed that for the same strains, rapid degradations of both enantiomers (1R-cis-bifenthrin and 1S-cis-bifenthrin) were attained with BF6 and BF28, whereas limited degradation was observed for the BF24 strain (Liu et al. 2005).

Three bacterial species reported to rapidly degrade permethrin in medium were identified as *Aeromonas sobria*, *Erwinia carotovora*, and *Yersinia frederiksenii*. Cis-permethrin degradation half-lives for these different bacteria ranged from 1.3 to 1.5 days (Liu et al. 2005).

Rare strains were reported to degrade cypermethrin in soil samples. A *Bacillus* sp. strain (ISTDS2) was described to completely accomplish this task but only after 30 days (Sundaram et al. 2013). Cypermethrin degradation was also tested in soil by the addition of *Pseudomonas* sp. Cyp19. Degradation of cypermethrin in un-autoclaved and autoclaved soil was 97.5% and 95% respectively after 30 days of incubation (Malik et al. 2009).

Regarding metabolite toxicity and impact on the environment, the goal of soil decontamination is not only the pesticides' degradation but also to accomplish complete mineralization. Additionally, some researchers show that *Ochrobactrum lupin* DG-S-01 (Chen et al. 2011a) degraded 90% of 3-PBAcid within nine days and *Sphingomonas* SP-SC-1 (Tang et al. 2013) completely degraded 3-PBAcid to phenol; catechol and 2-phenoxyphen within one day, in medium. *Streptomyces aureus* HP-S-01 was reported to completely degrade, in medium, the other main metabolite of SPs, 3-PBAld, after 6–7 days of incubation (Chen et al. 2011c). Complete mineralization of 3-PBAcid was achieved in soil by *Stenotrophomonas* sp. ZS-S-01 after 10 days (Chen et al. 2011d). A proven advantage of bacterial strains for metabolite elimination in soil was ascertained when using *Bacillus* sp. DG-02 inoculated in soil. It was observed that the $T_{1/2}$ for 3-PBAcid was greatly reduced (3.4–4.1 days) when compared to the non-inoculated control soil (101.9–187.3 days) (Chen et al. 2012a).

The biodegradation functionality of the microorganisms was mostly tested in ideal conditions (in medium with optimized temperature and pH) before its subsequent application *in situ*. The degradation potential of these strains, some without any toxic by products formed, revealed their significance as a biological agent for the remediation of pyrethroid soil contaminated sites.

Fungi

Fungi are important microorganisms that possess biochemical and ecological capacity to degradexenobiotic compounds (Harms et al. 2011). However the potential use for fungi in bioremediation of pyrethroids has not received the attention it deserves. To our knowledge, there are significantly less reports about bioremediation of pyrethroids by *Fungi* when compared with *Bacteria*.

Saikia and Gopal (2004) studied β-cyfluthrin degradation in Czapek dox medium by five fungi in pure culture (*Trichodermaviride* 5-2, *Trichoderma viride*

2211, *Aspergillus niger*, *Aspergillus terricola*, and *Phanerochaete chrysoporium*). The degradation half-lives for the different fungal strains were *T. viride* 5-2 7.07–14.14 days, *T. viride* 2211 10.66–46.20 days, *Phanerochaete chrysoporium* 18.73 days, *A. terricola* 38.50–77.07 days, and *A. niger* 57.75 days.

A fungus strain isolated from activated sludge was identified as a *Cladosporium* sp. HU and was found efficient for pyrethroid degradation in mineral salt medium. Fenvalerate, fenpropathrin, and β-cypermethrin were completely degraded after five days while deltamethrin, bifenthrin, and permethrin were degraded 94.6%, 92.1%, and 91.6% at the end of the experiment, respectively. The degradation half-lives of each substrate ranged from 0.99 to 1.54 days. The fungus hydrolyzed fenvalerate in the carboxylester linkage to produce α-hydroxy-3-phenoxy-benzeneacetonitrile and 3-PBAld and then degraded these two compounds (Chen et al. 2011b).

A novel yeast strain ZS-02, isolated from activated sludge and identified as *Candida pelliculosa*, was found highly effective in degrading bifenthrin over a wide range of temperatures and pH. Under the optimal conditions, the yeast completely metabolized bifenthrin within eight days (Chen et al. 2012c).

These described applications of fungi used as pyrethroid degrader make fungi suited for use in pyrethroid-contaminated environments.

Enzymes

Enzymes can also catalyze unspecific reactions and are actually recognized as promiscuous biocatalysts capable of transforming a variety of substrates that share structural similarity with their primary substrate (Aharoni et al. 2005; Khersonsky and Tawfik 2010). Some pyrethroid esterases have consistently been purified and characterized from various resources including metagenomes and organisms.

A novel pyrethroid-hydrolyzing enzyme Sys410 was recently isolated from Tuban Basin soil through a metagenomic approach. This enzyme efficiently degraded cyhalothrin, cypermethrin, sumicidin, and deltamethrin under assay conditions for 15 min, exceeding 95% hydrolysis efficiency (Fan et al. 2012).

A capable pyrethroid-hydrolyzing carboxylesterase was purified from mouse (Stok et al. 2004) and human (Nishi et al. 2006) liver microsomes. The carboxylesterases are enzymes that catalyze the hydrolysis of a wide range of ester-containing endogenous and xenobiotic compounds.

Enzyme extracts from bacterial and fungal strains were also reported as pyrethroid degraders. Purified enzyme from the fungus *Aspergillus niger* ZD11 was described to have a specific role on pyrethroid pesticides. Its hydrolysis action towards trans-permethrin was fastest, while deltamethrin was the least readily attacked. Cis-permethrin was hydrolyzed at an approximately equal rate as trans-permethrin (Liang et al. 2005). Pyrethroid detoxification was also achieved with a cell-free microbial enzyme system referred to as a *Bacillus cereus* SM3 permethrinase. Researchers think that this esterase seems to be a

carboxylesterase (Maloney et al. 1993). The pyrethroid-hydrolyzing esterase gene from *Klebsiella* sp. ZD112 was cloned and sequenced. There combinant gene encoding pyrethroid-hydrolyzing esterase (EstP) was purified and characterized. The purified EstP not only degraded many pyrethroid pesticides and the organophosphorus insecticide malathion, but also hydrolyzed F-nitrophenyl esters of various fatty acids, indicating that EstP is an esterase with broad substrate range. Trans- and cis-permethrin hydrolyzes indicate higher hydrolyzes efficiency than the carboxylesterases from resistant insects and mammals (Wu et al. 2006).

These pyrethroid-hydrolyzing enzymes could conceivably be developed to fulfill the requirements to enable its use *in situ* for detoxification of pyrethroids where they cause environmental problems.

Conclusion

Pyrethroids' wide use as insecticides in agriculture, veterinary, and domestic applications raises environmental concerns. Pyrethroid pesticides residues have been frequently detected in soils and thus in agricultural samples. The microbial breakdown of insecticides is considered by some authors as the most important catabolic reaction in soil and can be crucial when developing processes for pesticide decontamination.

Biodegradation reports showed that isolated microorganisms and mixed cultures were capable of proficiently degrading different SPs even when present as the sole carbon source. Bacteria, fungi, and enzymes possess potential for their use in bioremediation of pyrethroid-contaminated soils.

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1.3 Ecotoxicological effects of insecticides in plants assessed by germination and other phytotoxicity tools

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Statement of contribution

The contribution of the candidate, Idalina Bragança, in this work includes the literature review about soils contamination by insecticides, germination tests and germination tests as a tool to access phytotoxicity by insecticides, and the write of the book chapter regarding to the subjects researched.

Ecotoxicological Effects of Insecticides in Plants Assessed by Germination and Other Phytotoxicity Tools

Idalina Bragança, Clara Grosso, Diana Rede, Susana R. Sousa, Paulo C. Lemos, Valentina F. Domingues, and Cristina Delerue-Matos

Abstract The management of crop-pests relies largely on conventional insecticides. Farmers around the world use pesticides as an insurance policy against the possibility of a devastating crop loss from pests and diseases. Conversely, the use of insecticides has several drawbacks for agriculture, such as decrease in pollinator population and terrestrial pollution as they are frequently detected in the environment.

Several tests are used to assess phytotoxicity regarding several mechanisms affecting plants, namely, (a) inhibition of biological processes such as photosynthesis, cell division, enzyme function, and root, shoot, and leaf development; (b) interference with the synthesis of pigments, proteins, or DNA; (c) cell membrane instability; and (d) the promotion of uncontrolled growth. Germination tests are extensively used to assess the toxicity induced by pollutants. In these types of tests, the germination indexes and the seedling's growth and development are evaluated in a dose-response manner.

This review evaluates the application of insecticides leading to alteration on germination, in biochemical, physiological, and different enzymatic and nonenzymatic antioxidant levels that may affect the crop yield and insecticide residues in plants. As such, this chapter represents a systematic and integrated picture of insecticide toxicological effects on plants, highlighting germination.

Keywords Insecticides · Germination · Toxicology · Seeds · Bioassay

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1 Contamination of Soils by Insecticides

There is a myriad of relationships between environment and human health that are often more intricate than can be thought (Fig. 1). Since soil is a crucial component of the environment, studies aiming to evaluate terrestrial toxicity should consider the interactions between chemicals and soil in order to foresee the effect of chemicals on the environment (Kapanen and Itävaara 2001). As it is difficult to evaluate the effect of pollutants on the environment only based on the concentrations of chemical constituents, biotests are also needed (Kapanen and Itävaara 2001).

Agriculture represents an important sector in the worldwide economy. Therefore, the use of pesticides for pest management in agriculture is a common practice to maximize crop production. In the last decades, the use of pesticides has reduced crop losses caused by pests, but their intensive and large-scale application has caused several adverse effects on the environment by remaining in soils and water and by affecting the development of target and nontarget species. Due to adaptation and resistance developed by the target pests to pesticides, every year higher amounts and new chemical compounds are used to protect crops, causing undesired side effects and increasing the costs of food production (Carvalho 2006).

Insecticides are considered a quick, easy, and cheap solution for controlling insect pests; nonetheless, their use comes with a significant environmental cost. Studies included in this section were retrieved from Web of Science core database, between 2007 and 2017, using "soil contamination with insecticides" as a search topic (Table 1). Organochlorine pesticides (OCPs) were those more often found in the literature and also the ones detected in higher concentrations, despite their restricted use and even their production and usage banned in some countries. This type of insecticide is still often detectable in soil and persists in the environment due to their non-biodegradability or very slow degradation. More than half of the reports were

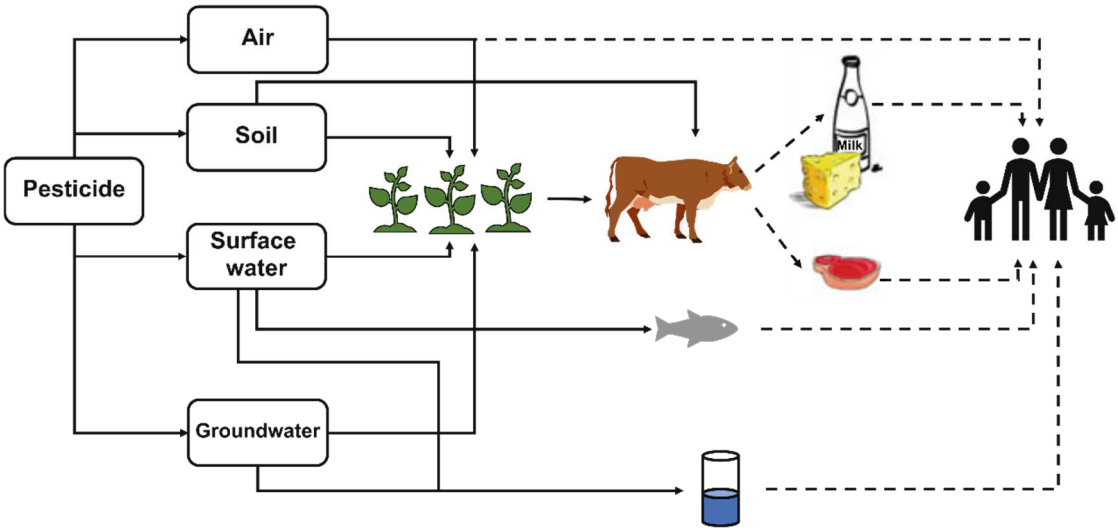

Fig. 1 Exposure routes generally considered in human exposure assessment (Adapted from Kapanen and Itävaara 2001)

Table 1 Residues of insecticides detected in soils/sediments collected from different areas of the planet

Continent	Area	Insecticides analysed	Sampling year	Concentration	References
Africa	Limpopo	DDTs (DDT+DDD+DDE)	February 2008	∑DDTs: village exposed: 5.7–59 μg/kg reference village: 2.1–93 μg/kg	Van Dyk et al. (2010)
	Togo	δ-hexachlorocyclohexane (HCH), heptachlor epoxide, 4,4-DDE, endosulphan (α, β and sulphate), λ-cyalothrin and chlorpyrifos	–	Concentrations of insecticides in different sites and farmers' plots varied from ND–26.93 μg/kg (dw).	Mawussi et al. (2014)
America	Canada	Neonicotinoids: clothianidin and thiamethoxam	April and May of 2014	Clothianidin in particulate matter from (mean value): oat: 9.91 μg/kg canola: 10.20 μg/kg Thiamethoxam: ND	Main et al. (2016)
	Costa Rica	Endosulfan I, endosulfan II, endosulfansulfate	February 2004	Σ Endosulfan (dw): 20–3175 pg/g	Daly et al. (2007)
	Mexico (Southern Sonora)	OCPs: benzene hexachloride (BHC or HCB), lindane, aldrin, endrin, β-endosulfan, methoxychlor, DDTs	2007	BHC: ND–938.5 μg/g Endrin: ND–377.3 μg/g DDTs: ND–679.7 μg/g	Cantu-Soto et al. (2011)
Asia	China	OCPs: HCHs, DDTs chlordane compounds and endosulfans OPs: chlorpyrifos, diazinon, dichlorvos, ethion, ethyl p-nitrophenyl (EPN), malathion, parathion-methyl, profenofos, trichlorfon, and terbufos OPYs: bifenthrin, λ-cyhalothrin, cyfluthrin, cypermethrin(CP), deltamethrin, esfenvalerate, fenpropathrin, permethrin, and tefluthrin Fipronil and its metabolites	1957–2012	Total insecticide concentrations in river sediments (dw): Rural: 67.6–1671 ng/g Suburban: 99.2–231 ng/g.	Sun et al. (2016)

(continued)

Table 1 (continued)

Continent	Area	Insecticides analysed	Sampling year	Concentration	References
	China	OCPs:δ-hexachlorocyclohexanes (HCHs), DDTs, chlorothalonil and dicofol OPs: methamidophos, methylparathion and parathion OPYs: fenvalerate (FEN)	–	Ten OCPs, three OPs and one OPYs detected: OCPs>OPs>OPYs OCPs and OPs concentration were <150 μg/kg. FEN concentration: 1227 μg/kg (maximal value of all pesticides detected).	Guo et al. (2016)
	China (sediments of Nanfei River and of cores from Chaohu Lak estuaries)	Eighteen OCPs: DDTs, HCHs, HCB, aldrin, endrin, heptachlor, heptachlor epoxide A/B, and tetrachloronitrobenzene	July and September of 2012	OCP concentrations: surface sediments: 3.48–121.08 ng/g core sediments: 0.60–39.28 ng/g	Zhang et al. (2016)
	China (Pearl River Delta)	OCPs: DDTs and HCHs OPYs: bifenthrin, fenpropathrin, tefluthrin, λ-cyhalothrin, permethrin, cyfluthrin, CP, esfenvalerate, and deltamethrin OPs: parathion-methyl, malathion, and chlorpyrifos	December 2009 to March 2010	Soil inventories of DDTs and HCHs were 100 and 83 tons, for pyrethroids and organophosphates 39 and 6.2 tons, respectively.	Wei et al. (2015)
	China (Guanting Reservoir area)	DDT, HCH and their metabolites	2003	ΣHCHs: 0.0 to 7.3 ng/g dw ΣDDTs: 0.0 to 76 ng/g dw	Wang et al. (2007)
	India (Unnao district)	OCPs: Aldrin, dieldrin, endrin, HCB, HCHs, DDTs, endosulfan isomers (α and β), endosulfansulfate, heptachlor and its metabolites, α andγ-chlordane and methoxychlor	October 2003	Σ DDT: b.d –74.06 ng/g Σ Endosulfan: b.d –13.07 ng/g ΣHCHs: 0.08 –7.25 ng/g Σ Chlordane: b.d –5.84 ng/g Σ Heptachlor: b.d –1.68 ng/g Σ OCPs: 0.36–104.50 ng/g	Singh et al. (2007)

India (Kuttanad agroecosystem)	OCPs: BHCs, heptachlor, chlordanes, endosulfans, aldrin, dieldrin and endrins	–	BHC: 0.01–9.55 ng/g DDT: ND–4.55 ng/g DDE: ND–2.18 ng/g DDD: ND–2.07 ng/g α-Endosulfan: 0.74–8.9 ng/g Aldrin: 1.96–2.73 ng/g Dieldrin: 1.29–3.72 ng/g Heptachlor: 1.01–8.52 ng/g	Sruthi et al. (2017)
India (North East)	OCPs: DDTs and HCHs	2009–2011	District Dibrugarh: 71.2–834 ng/g HCHs 30.1–918 ng/g DDTs District Nagaon: 39.2–743 ng/g HCHs 72.5–932 ng/g DDTs	Mishra et al. (2013)
India (Ranga Reddy)	Monocrotophos, chlorpyriphos, endosulfan and CP	2008–2009	α-endosulfan: 0.02 μg/g β-endosulfan: 0.02 μg/g	Ratna Kumari et al. (2012)
India (Nagaon and Dibrugarh)	OCPs: DDTs and HCHs	2009–2010	Nagaon: 98–1945 ng/g HCHs 166–2288 ng/g DDTs Dibrugarh: 178–1701 ng/g HCHs 75–2296 ng/g DDTs	Mishra et al. (2012)
India (Haryana)	OCPs: HCHs, DDTs, endosulphan, heptachlor, aldrin and chlordane OPYs: CP, fluvalinate, FEN, deltamethrin OPs: chlorpyriphos, monocrotophos, dimethoate, Me-parathion, palathion, quinalphos, triazophos	2002–2003	HCHs: 0.002–0.051 μg/g DDTs: 0.001–0.066 μg/g Endosulfan: 0.002–0.039 μg/g Chlordane: 0.0002–0.019 μg/g CP: 0.001–0.035 μg/g FEN: 0.001–0.022 μg/g Chlorpyriphos: 0.002–0.172 μg/g Malathion: 0.002–0.008 μg/g Quinalphos: 0.001–0.010 μg/g	Kumari et al. (2008)

(continued)

Table 1 (continued)

Continent	Area	Insecticides analysed	Sampling year	Concentration	References
	Japan	Dieldrin	–	0.068–0.125 mg/kg	Saito et al. (2012)
	Tajikistan	DDTs, BHCs, endosulfans, Aldrin, chlordanes, dieldrin, endrins, heptachlors and methoxychlor	2011, 2012, 2013 and 2014	Many soil samples with pesticides concentrations greater than 10 ppm.	Barron et al. (2017)
	Vietnam	DDTs, HCHs, chlordanes, drin compounds, heptachlor, HCB, heptachlor-epoxide and polychlorinated biphenyls (PCBs)	January to October 2002	ΣDDTs: 0.19–140 ng/g Σchlordanes: ND–9.0 ng/g ΣPCBs: 0.11–110 ng/g	Kishida et al. (2007)
Europe	France	Lindane (γ-HCH)	June 2002 to November 2007	γ-HCH: 0.03–4.92 μg/kg	Villanneau et al. (2009)
	Portugal and Spain (Agueda river borders)	Diazinon, chlorpyrifos and dimethoate	January–February 2012	None insecticide detected in soil.	Sánchez-González et al. (2013)
	Serbia (Belgrade)	Deltamethrin, phorate, fenitrothion, chlorpyrifos, carbofuran, γ-HCH, tebupirimfos and terbufos	July–November of 2006	Fenitrothion: b.d–80.5 μg/kg Chlorpyrifos: b.d–47.4 μg/kg	Marković et al. (2010)
	Spain (La Rioja region)	Methoxyfenozide and pirimicarb	March, June and October 2012	Methoxyfenozide: 4.61 μg/kg	Pose-Juan et al. (2015)
	Spain (Valencia)	OPYs: Resmethrin, bifenthrin, fenpropathrin, λ-cyhalothrin, permethrin, cyfluthrin, α-CP, τ-fluvalinate, esfenvalerate and deltamethrin	–	OPYs concentration ≤57.0 ng/g before plow and ≤62.3 ng/g during rice production. Resmethrin and cyfluthrin were the compounds found in higher concentrations.	Aznar et al. (2017)

ND non-detectable, *b.d* below detection limit, and *dw* dry weight

found in the Asian continent, mostly from India followed closely by China. These results could be explained by the high pattern of usage of insecticides in India compared with the rest of the world. In India, 80% of the pesticides used are insecticides, in contrast to 29.5% of world use (De et al. 2014). OCPs were massively used in China between 1983 and 2007 for pest and disease control, which explains their high residue level detection in recent years (Guo et al. 2016). Concerning insecticide concentrations, the highest amounts were found in Mexico, more precisely in Southern Sonora, for benzene hexachloride (BHC) and dichloro-diphenyl-trichloroethane (DDT) and its metabolites, dichlorodiphenyldichloroethane (DDD) and dichlorodiphenyldichloroethylene (DDE) in a range from non-detectable (ND) to 938.5 μg/g and ND to 679.7 μg/g, respectively. This study suggests evidence that although DDT was banned, it is still applied in Mexican agricultural soils (Cantu-Soto et al. 2011). Besides OCPs, other insecticide classes were found, namely, neonicotinoids, organophosphates (OPs), and pyrethroids (OPYs).

2 Tests to Assess Phytotoxicity by Insecticides

Pesticides are used globally for the protection of food, and more generally for human health, by killing organisms that cause disease and threaten public health. Insecticides are used to kill, prevent, repel, or harmfully affect insects. Thus, by nature, they present a degree of toxicity for the environment, plant, and animal species. At first thought, plants are not an insecticide target, however, they reach plant tissues when spread.

When an insecticide applied for insect control causes damage to the host-plant tissues, it is said to be phytotoxic. The susceptibility of plants to chemical injury varies greatly among and within species and is influenced by a myriad of factors, such as growth stage, growth rate, temperature, humidity, and other environmental factors (National Research Council 1969). Plants sensitive to harmful substances can be used as bioindicators in toxicity assessment studies, while more resistant plants can be applied in bioremediation processes (Kapanen and Itävaara 2001).

The use of bioindicators to evaluate possible phytotoxic chemical residues affords a direct, inexpensive, and integrated estimation of bioavailability and contaminant toxicity. The advantages of bioindicators consist of:

1. The possibility to detect both toxicity of parent compounds and toxic metabolites;
2. Tests use readily available materials;
3. Tests can be carried out ex situ or in situ;
4. The test period is usually short;
5. Low cost and uncomplicated methodology is used (Maila and Cloete 2005).

However, for environmental evaluation, tests also need to combine some other features, such as (1) to be standardized, (2) to have a defined endpoint, and (3) to be sensitive enough to distinguish differences among sites (Da Silva Júnior et al. 2013).

Phytotoxicity may be chronic, if it induces the immediate death of the affected plant tissue, or acute if it interferes with physiological processes that decrease the performance of the plant (National Research Council 1969). Phytotoxicity can be evaluated in several stages of plant development (seed germination, root elongation, and seedling growth) and can be carried out in pots or in Petri dishes. The inhibition of seed germination and the effects on root elongation or plant growth are the main areas of interest in studies on phytotoxicity, while photosynthesis, respiration, enzyme activities, and tissue cultures are not commonly used as standard tests (Kapanen and Itävaara 2001).

Several bioassay studies demonstrated that the use of insecticides can induce phytotoxic effects in different ways. First, natural pollinators, such as honeybees and butterflies, are very sensitive to pesticides. Pesticides can kill bees and are strongly implicated in pollinator decline (Miller 2004). Besides negatively affecting pollinator populations and their delivery of pollination, the use of insecticide may also have nonlethal impacts that distress the pollination process at pre- or post-pollen deposition stages. For instance, pesticides can make crops unattractive to a major pollinator, or negatively impact post-pollination processes, like pollen germination. Such impacts have received very little attention, and, given the potential for new insecticides to come into use, or for applications to increase in certain crops in response to emergent pests or diseases, a better understanding of these impacts is crucial (Gillespie et al. 2014).

Post-pollination impacts of pesticides could occur through pollen, stigmas, or the interaction of both. Either pollen or the stigmatic tissue may be susceptible to damage by pesticides, which can decrease pollen germination, pollen tube growth, and ovule fertilization, thus resulting in reduced seed set and crop yield (Gillespie et al. 2014).

Seed germination and root elongation tests (Fig. 2) have been used as short-term phytotoxicity test to provide valuable information about inhibition, enzyme activation, hormone production, cell expansion, respiration, and other parameters (Wang et al. 2001). This topic is worthy of a detailed discussion and a full view of the impact of insecticides in seed germination, and seedling development will be provided in Sect. 2.2.

Moreover, insecticides sprayed over the leaves also affect photosynthetic efficiency (Fig. 2) by increasing or decreasing pigment contents and affecting electron transport chain in chloroplasts. The pyrethroid insecticide alphamethrin was shown to induce a decline in chlorophyll a (chl a), chl b, and total chlorophyll in soybeans (*Glycine max* (L.) Merr) at higher concentrations, while carotenoid content increased with increasing concentrations of the insecticide, to protect chlorophylls from photooxidative damage. Regardless of the control group or insecticide-treated groups, the quantities of photosynthetic pigments were maximum at the flowering stage, followed by the pre-flowering and post-flowering stages. The highest concentration of insecticide tested also caused a reduction in the photosynthetic rate and stomatal conductance (Bashir et al. 2014). Chopade et al. (2007) quantified chl a and chl b in ten medicinal plants treated with one of three insecticides (endosulfan, lindane, and dichlorvos) and concluded that, in general, endosulfan was the most

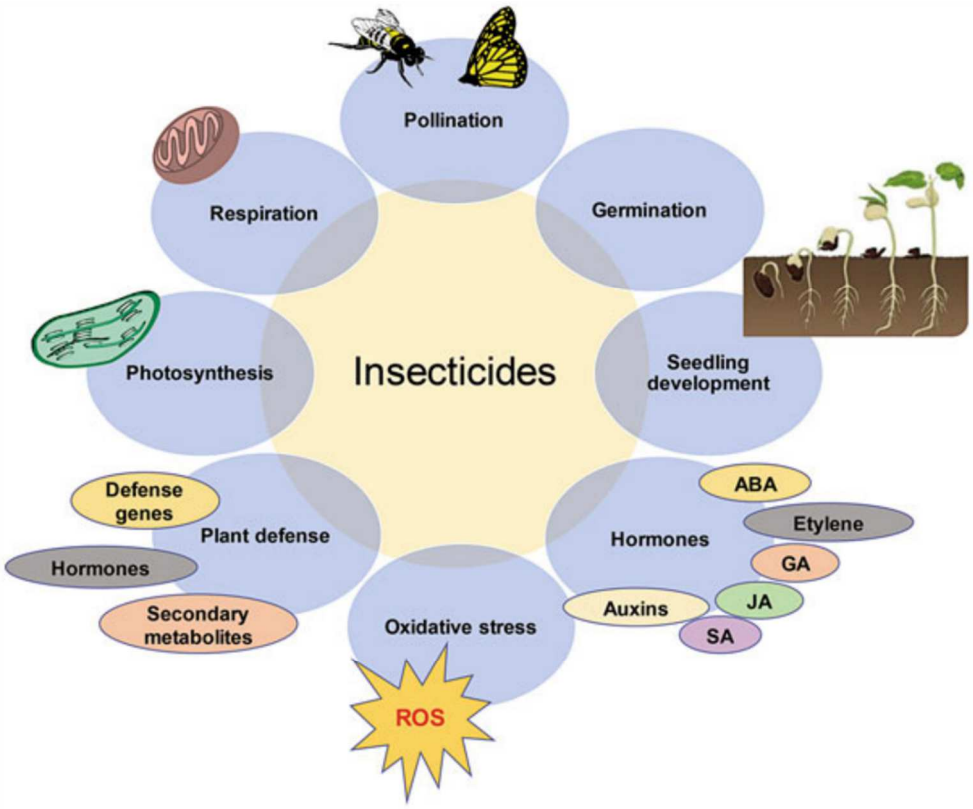

Fig. 2 Impact of insecticides on the development and survival of plant species. *ABA* abscisic acid, *GA* gibberellins, *JA* jasmonic acid, *ROS* reactive oxygen species, *SA* salicylic acid

harmful one. Reduction of photosynthetic pigments may be due to several mechanisms, namely, the inhibition of their biosynthesis or breakdown of pigments or their precursor molecules, changes in chlorophyll fluorescence associated with inhibition of electron transfer chain, the breakdown of the thylakoid and chloroplast envelope, and the decrease in leaf area (Mohamed and Akladious 2017). Chlorophyll breakdown in higher plants occurs in chloroplasts by enzyme-catalyzed processes via pheophorbide a and the red chlorophyll catabolite (RCC) to give primary fluorescent chlorophyll catabolite (*p*FCC). When possessing a propionic acid group, FCCs are translocated to the vacuole, where they spontaneously isomerize to the corresponding nonfluorescent chlorophyll catabolites (NCCs) (Chopade et al. 2007). The opposite effect was described in mung bean (*Vigna radiata* (L.) Wilczek) submitted to foliar spray application of dimecron. Chlorophyll content increased but a deviation in the absorption spectra of chl a and chl b was observed (Siddiqui and Khan 2001). Possible explanations suggested for this phenomenon could be related with one of the following phenomena, the increase of grana and intergrana spaces, NADP/NAD ratios, and NADP and ATP levels, or could be due to increased uptake of K^+, Mg^+, Ca^{2+}, or other ions (Mohamed and Akladious 2017).

Besides interfering with the photosynthesis, insecticides also affect photo and dark respiration in seedlings (Fig. 2) (Mishra et al. 2008). Seedlings of cowpea (*Vigna unguiculata* (L.) Walp.) exposed to higher doses of dimethoate and/or UV-B

showed reduced content in chl a and chl b and carotenoids, as well as photosynthetic oxygen yield and photofixation of CO_2. This could be explained based on the direct effect of dimethoate on the activities of photosystems II, I, and whole electron transport chain in chloroplasts which were inhibited by the insecticide or insecticide+UV-B in a concentration-dependent manner (except at the lower dose of dimethoate tested). Similarly, high doses of insecticide and insecticide+UV-B adversely affected photo and dark respiration, probably due to the interaction of dimethoate and UV-B with ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) and other enzymes involved in photorespiration. Opposite effects were verified for low doses of dimethoate.

Application of insecticides can also suppress the expression of important plant defense genes, alter levels of phytohormones involved in plant defense, and decrease plant resistance to unsusceptible herbivores (Fig. 2). Szczepaniec et al. (2013) applied thiamethoxam, clothianidin, and imidacloprid to three different crops, cotton (*Gossypium hirsutum* L.), corn (*Zea mays* L.), and tomato (*Lycopersicon esculentum* Mill.), respectively. The expression of genes coding for phenylalanine ammonia lyase (*PAL*), coenzyme A ligase (*CoA ligase*), and chitinase (*chit*), involved in salicylic acid (SA)-mediated defense, and trypsin proteinase inhibitor (*trypsin PI*), involved in jasmonic acid (JA)-mediated defense, was influenced by the treatment with spider mites and/or insecticide. Spider mites induced the expression of *CoA ligase* and *chitinase* in cotton and elicited the expression of all four genes in corn. *Trypsin PI* was the only gene induced in tomato. When treated with insecticide or insecticide+mites, the expression of *CoA ligase* and *chitinase* increased in cotton exposed to both treatments while none of the genes were induced in corn. Both treatments strongly induced *chitinase* expression in tomato. Besides altering the expression of defense genes, insecticide treatments also changed phytohormone concentrations in these species. All insecticide decreased the levels of 12-oxo-phytodienoic acid (OPDA), a precursor of JA, and imidacloprid enhanced the level of SA and decreased the levels of JA and of a bioactive conjugate of JA (JA-Ile) in tomato. Clothianidin reduced the concentration of abscisic acid (ABA), a hormone also related to the plant defense, and JA in corn (Szczepaniec et al. 2013).

Foliar application of insecticides was shown to induce oxidative stress (Fig. 2). For instance, pre-flowering, flowering, and post-flowering seedlings of mung beans (*Vigna radiata* L.) were treated with chlorpyrifos, and lipid peroxidation rate, proline content, enzyme activity (superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and glutathione reductase (GR)), and ascorbate and glutathione content were determined (Parween et al. 2012). The increase of lipid peroxidation and proline content was age- and dose-dependent. Proline is known to contribute to detoxification of reactive oxygen species (ROS), protection of membrane integrity, stabilization of enzyme or proteins, and tolerance to stresses. In order to detoxify the superoxide radical anion and hydrogen peroxide produced, SOD, APX, CAT, and GR activities increased dose-dependently with maximum activity in the flowering stage, and, after this period, their activity declined. The relatively low activity during post-flowering stage is mainly based on the fact that an older leaf

contains lower antioxidants than a younger leaf. APX and GR are components of the ascorbate-glutathione cycle responsible for the recycling of glutathione, and, in this way, they protect chloroplasts by maintaining high reduced/oxidized glutathione (GSH/GSSG) ratio. Similar results were obtained by Bashir et al. (2007) for soybean seedlings treated with deltamethrin.

Concerning the nonenzymatic antioxidants, the decline in the ratio ascorbate/dehydroascorbate is indicative of oxidative stress. Chlorpyrifos induced a dose-dependent reduction in ascorbate as well as in ascorbate+dehydroascorbate contents over time in *V. radiata*, along with a switch of the ratio favoring dehydroascorbate. Concerning GSH, a dose-dependent decrease in GSH and an increase in GSSG and GSH + GSSG contents were observed (Parween et al. 2012). However, in another study, deltamethrin induced an increase of GSH in soybean seedlings, indicating an active GSH participation in the detoxification of ROS, directly (nonenzymatic) as well as through certain enzymes (Bashir et al. 2007).

The synthesis of secondary metabolites in plants, such as phenolic compounds, can be modulated by the application of insecticides. Increase of polyphenol content was observed for mung bean seedlings exposed to profenofos (Mishra et al. 2015) and dimecron (Siddiqui and Khan 2001). These compounds are important for plant defense since they can be toxic for plant pathogens and can also protect plants from oxidative stress.

Therefore, to avoid the negative impact at all levels of the ecosystem, research on insecticides with fewer side effects should be encouraged, and pesticides manufacturers should conduct long-term studies on target and nontarget species to demonstrate that a pesticide has no adverse effects before allowing it to be registered for use in the environment.

2.1 Germination Test

According to the International Rules for Seed Testing (ISTA 1966), germination in a laboratory test is defined as the emergence and development of essential structures from the seed embryo that, for the kind of seed tested, indicate its ability to develop into a normal plant under favorable soil conditions. In other words, it evaluates not only the germination process itself but also the seedling (root and shoot elongation) development. Germination and seedling development of several plant species demonstrated to be affected by insecticide exposure. Studies included in this section were obtained from Web of Science core database, between 2003 and 2017, using "seeds, germination, and insecticides" as a search topic (Table 2).

Some of these bioassays are ruled by specific standards and recommended for soil quality assessment. OECD has developed a plant bioassay (OECD 2006), intended to assess the potential effects of substances on seedling emergence and growth. It does not cover chronic effects or effects on reproduction, such as seed set, flower formation, and fruit maturation. The test assesses the effects on seedling and early growth of higher plants after exposure to the substance in the soil or in another soil

Table 2 Influence of insecticide application on seed germination and seedling development

Plant	Insecticides	Range	Germination conditions	Effect on germination	Other effects	Reference
• Alliaceae						
Allium cepa L.	λ –cyhalothrin, Spinetoram Methomyl Acetamiprid Spirotetramat Azadirachtin Urea	–	Field experiment	Insecticides have positive effects on the rate of seed germination. More seeds from treated plants germinated within 5 days.	Decreased flower visitation by honey bees. Can negatively affect multiple stages of the pollination process.	Gillespie et al. (2014)
Allium cepa L.	Me-parathion* *combined with lead, iron, cadmium, chrome, atrazine and 2,4-DB	–	Seeds exposed to sediments elutriatein Petri dishes.	–	Chromosomal aberrations and nuclear abnormalities in roots.	Rambo et al. (2017)
Allium porrum L.	Endosulfans DDTs Diedrin	OCPs mean concentrations (dw) Bulk soil: 36.4 ng/g Rhizosphere 13.7 ng/g	Field experiment	–	Aerial and root tissues bioaccumulate OCPs. High levels of metabolites found in all tissues suggest the ability to metabolize parent compounds.	Gonzalez et al. (2003)
• Asteraceae						
Lactuca sativa L.	Chlordanes mixture (*cis*-chlordane, *trans*-chlordane and *trans*-nonachlor and *p, p*'-DDE)	100 ng/mL	–	A statistically non-significant seed germination decrease was noted.	–	Hamdi et al. (2015)

• Brassicaceae						
Brassica rapa var. *chinensis* (L.) Kitamura	CP* * Alone or in combination with Cu^{2+}	Solution (mg/mL): CP: 5.6–17 Cu^{2+}: 1–9; CP + Cu^{2+}: 8 and 16 of CP + 1–9 of Cu^{2+} Soil (mg/kg): CP: 8– 64 Cu^{2+}: 100–300 CP+Cu^{2+}: 50 and 100 of CP+100–300 of Cu^{2+}	Petri dishes with contaminant solutions, vitreous pots with solutions and culture dishes with contaminated soil.	CP reduced germination and root and shoot elongation in solution. Cu^{2+} accelerated germination rate at low doses and inhibited it at high concentrations. CP reduced Cu^{2+} toxicity on seed germination and alleviated Cu^{2+} toxicity on root elongation at low concentrations. Under soil conditions, root and shoot elongation was practically unaffected by CP. CP + Cu^{2+} had less impact on root elongation than that of Cu^{2+} alone.	–	Liu et al. (2009)
Brassica sp.	HCHs	300–12500 mg/kg of total HCHs	Plastic pots containing soil.	Decrease in germination at the highest concentrations of the HCHs. Root length decreased at 10000 mg/kg. SVI: 100< SVI<300 SRL: 50<SRL<100 HCHs induced low productivity and biomass accumulated mainlyin root.	–	Pereira et al. (2010)

(continued)

Table 2 (continued)

Plant	Insecticides	Range	Germination conditions	Effect on germination	Other effects	Reference
Capsella bursa-pastoris L.	Deltamethrin (Decis) Dimethoate (Dimethoate 40)	25 g/L 400 g/L	Pots in controlled greenhouse environment.	No significant effect on germination. Only deltamethrin decreased seedling growth (mean dry weight biomass).	–	Hanley and Whiting (2005)
Raphanus sativus L.	Landfill leachate (LLe) contaminated with Dimethoate and other contaninants* * linuron, bisphenol A, 17α-ethynilestradiol 4-n-nonylphenol.	10 mg/L	Petri dishes with: non-contaminated LLe (NC); contaminated LLe (UN); LLedecontaminated by adsorption and/or ligninolytic fungi.	None of the treatments affected seed germination. UN mainly affected root and shoot elongation while NC inhibited only root elongation. LLe treated with adsorbents or with adsorbents+fungi increased elongation and seedling biomass.	–	Loffredo and Castellana (2015)
• Caryophyllaceae						
Agrostemma githago L.	Deltamethrin (Decis) Dimethoate (Dimethoate 40)	25 g/L 400 g./L	Pots in controlled greenhouse environment.	Insecticide application had no significant effect on germination. Only dimethoate decreased seedling growth (mean dwbiomass).	–	Hanley and Whiting (2005)
• Convolvulaceae						
Ipomea aquatica Forssk	Endosulphansulfate Heptachlor	0.4–40 mg/kg dry soil	Plastic planting containers with contaminated soil.	None of the insecticides significantly decreased seed germination and root length. Both insecticides at 40 mg/kg reduced fresh weight but not dw.	–	Somtrakoon and Pratumma (2012)

• Curcubitaceae						
Cucumis sativus L.	Endosulphansulfate Heptachlor	0.4–40 mg/kg dry soil	Plastic planting containers with contaminated soil.	None of OCPs significantly decrease seed germination or fresh and dw. Endosulphansulfate and heptachor at 40 mg/kg decreased shoot and root length, respectively.	–	Somtrakoon and Pratumma (2012)
Cucumis sativus L.	Permethrin	0.07–0.52 mg/mL	Filter paper hydrated with aqueous suspensions in Petri dish.	Nanopermethrin did not affect germination and root length but permethrin dose-dependently affected these parameters.	–	Kumar et al. (2013)
• Fabaceae						
Cicer arietinum L.	Endosulfan* Chlorpyrifos* *alone or combined with fungicide Captan and *Rhizobium* inoculant.	15 mL/kg 10 mL/kg	Field experiment, conducted at a research farm	No adverse effect on germination were found for Endosulfan and Chlorpyrifos when applied alone or in combination with the recommended fungicide Captan and *Rhizobium* inoculant.	Non-significant differences to controls were observed in nodulation, yield-attributing characters and grain yield.	Cheema et al. (2009)
Glycine max L.	CP (Arrivo 25 EC)	9.6 and 19.2 M	Petri dishes.	Germination percentages of primary roots decreased with increasing CP concentration.	Reduction in the number of different phases of mitosis and in the leaf pigment content.	Aksoy and Deveci (2012)

(continued)

Table 2 (continued)

Plant	Insecticides	Range	Germination conditions	Effect on germination	Other effects	Reference
Glycine max L.	Mepthion (fenitrothion) Myungtaja (etofenprox) Actara (thiamethoxam) Stonate (λ-cyhalothrin + thiamethoxam)	2 mL/L 1 mL/L 0.5 g/L 0.5 g/L	Petri dishes inside an incubator.	Fenitrothion reduced the seed germination, seed and seedling vigour.	Thiamethoxam and λ-cyhalothrin + thiamethoxam showed positive effects on seedling biomass and contents of phenolic compounds. Fenitrothion reduced polyphenol and flavonoid contents.	Dhungana et al. (2016)
Trifolium pratense L. *Trifolium repens* L. *Pisum sativum* L. *Phaseolus vulgaris* L.	HCHs	0–5000 mg/kg soil	Plastic pots containing soil	Germination was not affected as other parameters such as the rate of germination and SVI.	–	Pereira et al. (2010)
Senna obtusifolia (L.) H.S. Irwin & Barneby	Acephate* Carbaryl* Methomyl* Esfenvalerate* Fenpropathrin* λ-Cyhalothrin* Indoxacarb* *Mixture with 2,4-DB herbicide	1.09 kg/ha 1.12 kg/ha 0.67 kg/ha 0.20 kg/ha 0.34 kg/ha 0.36 kg/ha 0.12 kg/ha	Field experiment at a Cherry Farm Unit.	Seed production was not affected by co-application of the 2,4-DB with any of the insecticides to flowering sicklepod.	–	Lancaster et al. (2005)
Vigna sinensis L Savi	Endosulfansulfate Heptachlor	0.4–40 mg/kgdry soil	Plastic planting containers with contaminated soil.	The insecticides did not affect seed germination (%). Heptachlor affected shoot and root length more than endosulfansulfate.	–	Somtrakoon and Pratumma (2012)

• Poaceae						
Avena fatua L	Dimethoate Deltamethrin	40, 400 g/L 25 g /L	Pots filled with sterile compost in controlled greenhouse environment.	No significant effect was detected on seed germination.	–	Hanley and Whiting (2005)
Avena sativa L.	HCHs	300–12500 mg/kg of soil	Plastic pots containing soil	For concentration of 300 mg/kg it was observed an increase in germination but for highest concentrations there was a reduction. Reduction of the mean root length was observed. Shoot length, decreased above 1250 mg/kg.	–	Pereira et al. (2010)
Brachiaria brizantha Stapf. cv. Piatã	Thiamethoxam	17.5–40.0 g per 100 kg of seeds	Pots filled with a substrate composed of clay, silt and sand in controlled greenhouse environment.	No effect was detected on seed germination and growth. Increasing doses provoked a slight linear decrease of leaf area and a slight decrease in shoot development. Root growth tends to enhance.	The % of crude protein in the shoots increased with the increased availability of nitrogen in the plant. The activity of nitrate reductase increased.	Macedo et al. (2013)

(continued)

Table 2 (continued)

Plant	Insecticides	Range	Germination conditions	Effect on germination	Other effects	Reference
Cenchrus setigerus Vahl	Chlorpyrifos CP FEN	10–100 mg/kg dry soil	Plastic pots containing soil	Germination rates showed a tendency to decline with pesticide increasing concentration. Higher concentrations of chlorpyrifos caused a significant reduction and delay in seed germination comparatively to CP and FEN.	–	Dubey and Fulekar (2011)
Hordeum vulgare L.	HCHs	300–12500 mg/kg of soil	Plastic pots containing soil.	Reduction in germination (%) for HCHs highest concentrations. Shoot mean length, decreased above 1250 mg/kg.	–	Pereira et al. (2010)
Hordeum vulgare L. cv. karan-16	Chlorpyrifos	0.05 – 0.5%	Seeds were pre-treated with chlorpyrifos and placed on filter paper in Petri dishes.	Seed germination decreased with the increasing of dosage, being the effect more marked for 0.5% of treatment at a duration of 17 h pre-soaking (about 33.8%).	Reduced mitotic index and a gradual increase in % of chromosomal abnormalities for increasing concentrations. Reduction in chlorophyll for the highest concentrations. Carotenoid content slightly increased at 0.05% and 0.1%.	Dubey et al. (2015)

Hordeum vulgare L. cv. karan-16	Profenophos	0.05 - 0.5%	Seeds were pre-treated with profenophos and placed in Petri dishes.	Percentage of seed germination decreased with the increasing of concentration.	Chromosomal abnormalities and induction on chlorophyll mutations were observed.	Srivastava and Singh (2009)
Oryza sativa L.	Diazinon Fipronil λ-cyhalothrin	10 µg/L	Seeds were pre-treated with insecticides and placed on filter paper in Petri dishes.	Fipronil provoked a stimulatory effect onradicle growth.	–	Moore and Kroger (2010)
Oryza sativa L.	Imidacloprid (Gaucho® 600 FS)	250–2000 mg/L	15 cultivars of rice seeds were sown in translucent HDPE trays filled with soil.	Reduction of seed germination provoked by the continuous exposure to 2000 mg/L of imidacloprid. Concentrations of 500 –1000 mg/Lled to an enhancement of seedling growth.	–	Stevens et al. (2008)
Pennisetum pedicellatum Trin.	Chlorpyrifos CP FEN	10–100 mg/kg dry soil	Plastic pots containing soil.	Germination rates showed a tendency to decline with pesticide increasing concentration. Higher concentrations of chlorpyrifos caused a significant reduction and delay in seed germination comparatively to CP and FEN.	–	Dubey and Fulekar (2011)

(continued)

Table 2 (continued)

Plant	Insecticides	Range	Germination conditions	Effect on germination	Other effects	Reference
Poa annua L.	Dimethoate Deltamethrin	40, 400 g/L 25 g/L	Pots filled with sterile compost in controlled greenhouse environment.	No significant effect was detected on seed germination. Deltamethrin caused a reduction on seedling growth.	–	Hanley and Whiting (2005)
Triticum aestivum L.	HCHs	300 – 12500 mg/kg of soil	Plastic pots containing soil.	Decrease in total germination with the increasing concentrations of HCHs. The effect was more marked >5000 mg/kg. Reduction in the roots mean length was observed. Shoot mean length decreased >1250 mg/kg.	–	Pereira et al. (2010)
Zea mays L.	Permethrin	0.07 – 0.52 mg/L	Filter paperhydrated with aqueous suspensions in Petri dishes.	Permethrin significantly decreased germination (%) and root length. Nanopermethrin caused no effect on seed germination and root length.	–	Kumar et al. (2013)

Zea mays var. ceratina	Endosulfansulfate Heptachlor	0.4 – 40 mg/kg dry soil	Plastic planting containers with contaminated soil.	The insecticides did not cause a significant decrease on seed germination. Shoot length was stimulated by the lowest concentrations of insecticides. Fresh and dw increased in the range 0.4-4.0 mg/kg of endosulfansulfate. No significant combined effect on the root and shoot elongation was observed.	–	Somtrakoon and Pratumma (2012)
Zea mays L. *Saccharata* Sturt.	Endosulfansulfate Heptachlor	0.4 – 40 mg/kg dry soil	Plastic planting containers with contaminated soil.	Single toxicants did not cause a significant decrease of seed germination. The highest concentration of heptachlor provoked a reduction in root length. No combined effect on the root and shoot elongation and fresh and dw were observed.	–	Somtrakoon and Pratumma (2012)

(continued)

Table 2 (continued)

Plant	Insecticides	Range	Germination conditions	Effect on germination	Other effects	Reference
Zea mays L. *saccharata* Sturt.	Pyriproxyfen	0.1 – 0.6 ppm	Seeds were pre-treated with pyriproxyfen and placed in Petri dishes with two layers of filter paper.	Germination % decreased with increasing doses of insecticide. Elongation of radicle decreased and was almost inhibited at the highest level of pyriproxyfen. At 0.6 ppm the number of radicles and coleoptile length decreased.	Increasing of pyriproxyfen concentration: decreased the number, width and length of stomata and number of epidermal cells; decreased total chlorophyll and carotenoids; and increased anthocyanin and proline contents. Stomata indexes for both leaf surfaces were affected.	Coskun et al. (2015)
Zea mays L. *saccharata* Sturt.	Deltamethrin	0.01 – 0.5 ppm	Seeds were pre-treated with deltamethrin and placed in Petri dishes with two layers of filter paper.	Germination % decreased with increasing doses of deltamethrin. The length of radicle decreased being the effect more pronounced for the highest concentration.	Increasing of deltamethrin concentration: decreased stoma index and sizes; decreased chlorophylls a and b and carotenoid contents; and increased anthocyanin and proline contents.	Duran et al. (2015)
• Solanaceae						
Lycopersicon esculentum L.	Emamectin benzoate α-CP λ-cyhalothrin Imidacloprid	10 – 160 mg/L 30 – 500 mg/L 15 – 240 mg/L 125 – 2000 mg/L	Seeds were pre-treated with insecticides and placed in Petri dishes with two layers of filter paper.	Germination % and root growth decreased with increasing doses of insecticide. The inhibitory effectdecreased with the exposure time.	Chlorophylls a and b decreased with increasing concentration of pesticides.	Shakir et al. (2016)

				Longest shoots were observed with 60 mg/Lof α-CP while the smallest ones were caused by 500 mg/Lof α-CP. Biomass increased with lower concentrations and decreased with higher concentrations for all the insecticides.	Total carotenoid content was stimulated by lower concentrations of pesticides, and decreased with higher concentrations.	
Lycopersicon esculentum L.	Permethrin	0.07 – 0.52 mg/L	Filter paperhydrated with aqueous suspensions in Petri dishes.	Permethrin caused a decrease in both germination % and root length, being the effect more pronounced for 0.52 mg/L. Nanopermethrin caused no effect on seed germination and root length.	–	Kumar et al. (2013)
• Urticaceae						
Urtica urens L.	Dimethoate (Dimethoate 40 and BASF) Deltamethrin (Decis and Aventis)	40 and 400 g/L 25 g/L	Pots filled with sterile compost in controlled greenhouse environment.	No significant effect was detected on seed germination. The combination of both insecticides caused a reduction on seedling growth.	–	Hanley and Whiting (2005)

SVI seedling vigour index, *SRL* specific root length index, and *dw* dry weight

matrix. Seeds in the treatment groups and in the control group are placed in the respective soils, treated or untreated with the test compound, and the evaluation of the effects following around 14–21 days after 50% emergence of the seedlings in the control group is carried out. Endpoints measured in this test include the visual observation of seedling emergence, the measurement of dry shoot weight (alternatively fresh shoot weight) and, in some cases, of the shoot height, as well as an evaluation of visible damaging effects on different parts of the plant. These quantifications and observations are compared to untreated control plants.

Usually, insecticides can influence seed germination by affecting the synthesis and activity of hydrolytic enzymes, such as amylase, ATPase, lipase, and protease (Bashir et al. 2014). Also, the levels of phytohormones involved in germination can be altered. The application of phorate on seeds of mash bean (*Vigna mungo* (L.) Hepper) showed that lower concentrations of insecticide had a stimulatory effect on seed germination, accompanied by amylase activity and increased levels of the phytohormones gibberellin and ethylene, while higher concentrations reduced them (Singh et al. 1982).

Reduction in length of roots and shoots also occurs due to the application of insecticides, and this may be explained due to inhibition of cell division at the meristematic regions (Bashir et al. 2014; Dubey et al. 2015). Soumya et al. (2016) observed that even exposure of onion seeds (*Allium cepa* L.) to relatively low concentrations of the broad-spectrum insecticide Attack had significant effects on mitotic index and structure of chromosomes and disturbed the mitotic spindle formation. Similar results were obtained for the same species with the application of emamectin benzoate and imidacloprid (Al-ahmadi 2013) and for barley (*Hordeum vulgare* L.) treated with chlorpyrifos (Dubey et al. 2015). Mitotic index reduction may occur during the interphase due to (1) inhibition of DNA synthesis at the S phase; (2) blocking of the G_1 phase, thus suppressing the DNA synthesis; or (3) blocking of the G_2 phase, resulting in the prevention of cells from entering the mitosis. The most frequent chromosomal aberrations include chromosome fragmentation, chromosome stickiness, chromosomal bridges, diagonal anaphase, and multipolar anaphase (Dubey et al. 2015). Moreover, the reduced root elongation may also be correlated with the inefficient uptake of the nutrients from the soil (Bashir et al. 2014) or decrease in respiration rate (Lichtenstein et al. 1962).

Seedling development can also be impaired by insecticides that influence the nitrogen metabolism. Mathur et al. (1989) studied the influence of phorate on the activities of the enzymes glutamine synthetase (GS), glutamate dehydrogenase (GDH), and glutamate synthase (GOGAT) in the primary leaves, nitrogenase (N_2-ase) in detached root nodules, and protein concentration in the primary leaves of *V. mungo*. The authors observed that low concentrations of phorate stimulated the activities of these enzymes and protein concentration but behaved in the opposite way at higher concentrations.

To avoid seed destruction, insecticide or fungicide addition is commonly used. However, the addition of these chemicals showed significant lower germination percentages throughout the storage period, with a significant reduction after 8 months. However, it seems that encrusting seeds reduce the negative effect of pesticides on sunflower germination (Szemruch and Ferrari 2013).

From Table 2, it can be seen that most of the studies were performed with species belonging to Poaceae (41.5%) and Fabaceae (22.0%), followed by Brassicaceae (9.8%), Alliaceae (7.3%), Cucurbitaceae (4.9%), Solanaceae (4.9%), Asteraceae (2.4%), Caryophyllaceae (2.4%), Convolvulaceae (2.4%), and Urticaceae (2.4%) (Fig. 3A). Regarding the classes of insecticides tested by plant family, OPYs, OCPs, and OPs were the most used ones (Fig. 3B).

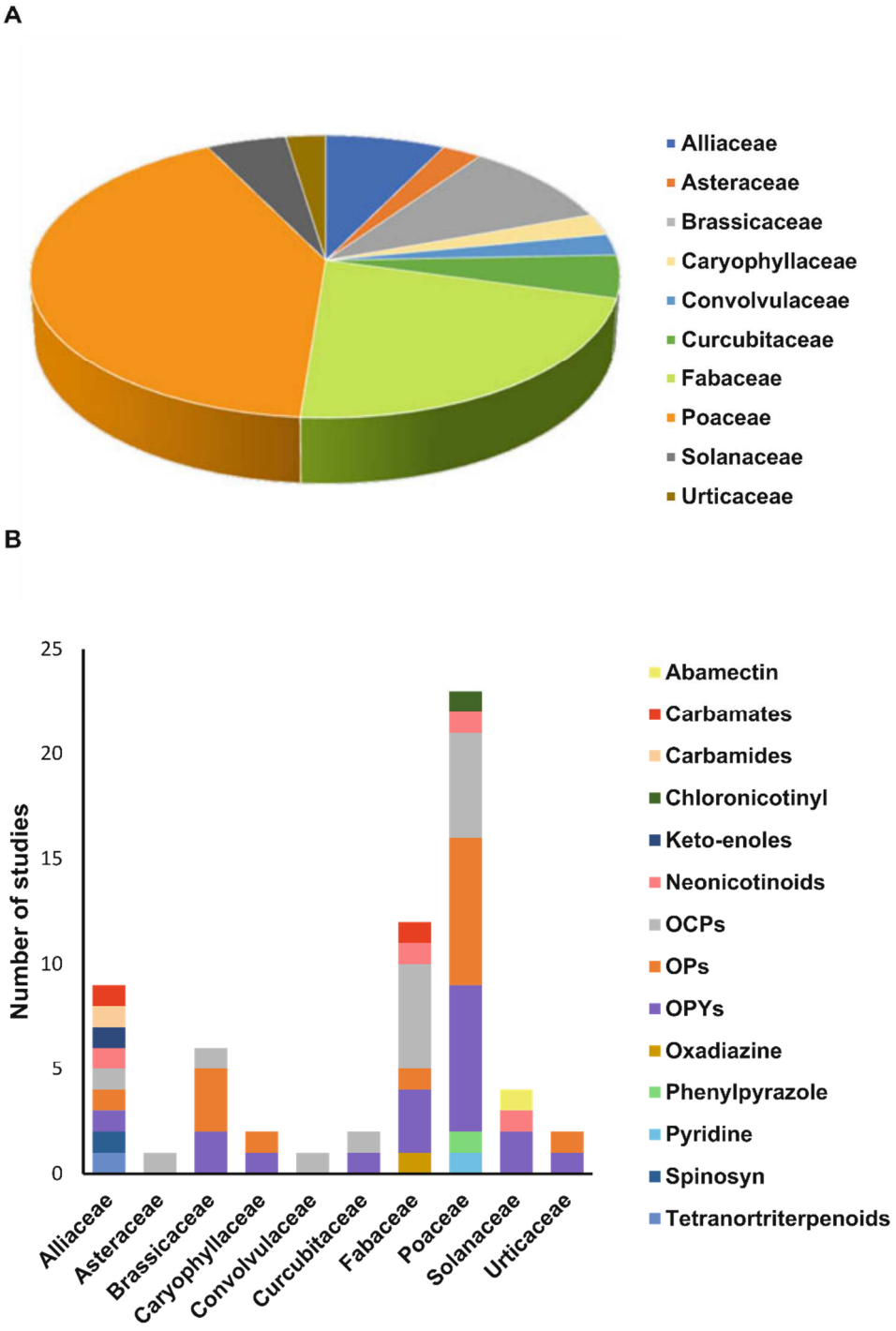

Fig. 3 Prevalence of plant species and insecticides used in germination tests. (**A**) Number of species by plant family, (**B**) classes of pesticides by plant family

2.2 *Germination Tests As a Tool to Assess Phytotoxicity by Insecticides*

The use of higher plants in ecotoxicological studies has increased in the past years since acute germination tests for testing pollutants are fast, sensitive, and cost-effective. Seeds are self-sufficient which means that there is no need to add nutrients to the samples (Wang and Freemark 1995). Thus, the maintenance cost of these tests is minimal and no major equipment is required. Seeds are highly sensitive to pollutants, and the germination test is simple, reproducible, and quick and can be applied in the laboratory (filter paper, Petri dish, and artificial soils) or in field experiments. Moreover, seeds are easily purchased and continue viable for an extended period of time (Wang and Freemark 1995; Priac et al. 2017). Therefore, the application of seed germination, root elongation, and early seedling growth tests to monitoring and assessing environmental conditions has more than a few advantages over animal toxic tests (Wang and Freemark 1995). The most common species recommended by OCDE (OECD 2006) and US Environmental Protection Agency (USEPA 1996) are *Cucumis sativus* L., *Lactuca sativa* L., *Raphanus* spp., *Trifolium pratense* L., *Brassica oleracea* L., and *Triticum aestivum* L., but as it can be seen in Table 2, several other species can be used as test organism. Otherwise, the response to pollutants is closely related to each plant species, and germination period can be insufficient to properly assess phytotoxicity. In field experiments, edaphic factors (e.g., minerals, pH, or salinity) related with soil and sediment can interfere with the toxic effects of the pollutant leading to difficult result interpretation (Wang and Freemark 1995).

3 Final Remarks

From all the classes of insecticides available on the market, OCPs were those more frequently found in the literature. Additionally, OCPs were also detected in higher concentrations in soil, regardless their production and usage banished in some countries. The number of studies demonstrating soil contamination by insecticide application occurs with more incidence in India and China. On the other hand, Mexico showed the highest concentrations of contaminants in soil.

Despite the benefits of insecticides killing or repelling harmful insects, they show a degree of toxicity for both environment and living organisms. Plants are not an insecticide target, but they can target plant tissues when applied, thus impacting their survival and normal development. Moreover, their effects can also negatively impact human beings by spreading along the food chain.

Bioassays allow a global assessment of toxicity, with germination assays being the most useful ones for soil quality evaluation and monitoring. Among their several advantages, minimal maintenance cost and the fact that no major equipment is required can be mentioned. Moreover, seeds are highly sensitive to pollutants;

the methodology is simple, reproducible, and quick and can be applied in situ or in vitro, requiring small amounts of sample.

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CHAPTER 2

Pyrethroid pesticides in soils

2.1

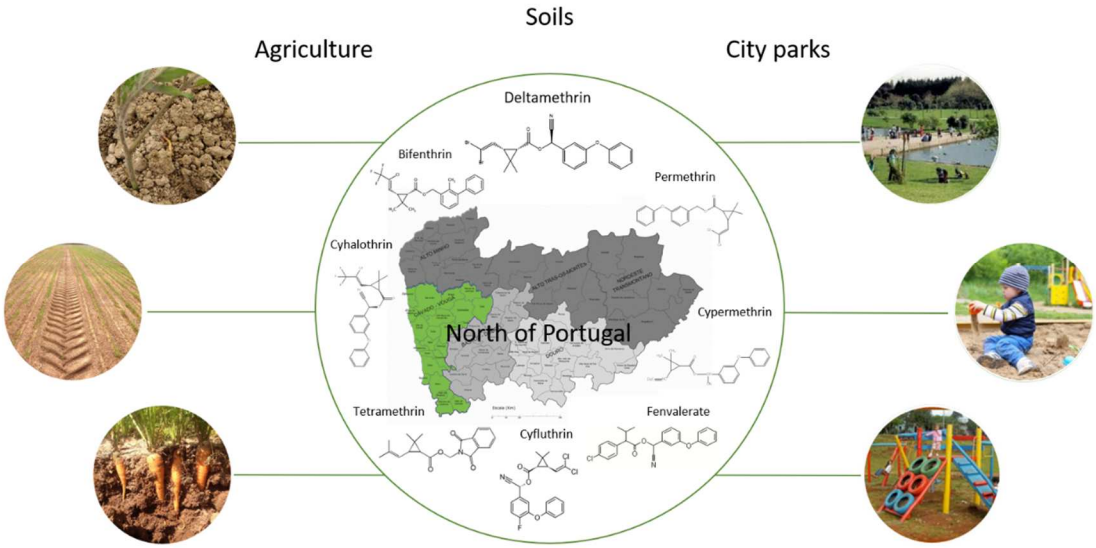
Assessment of pyrethroid pesticides in topsoils of northern Portugal

2.2

Pyrethroid pesticide metabolite, 3-PBA, in soils: method development and application to real agricultural soils

2.1 Assessment of pyrethroid pesticides in topsoils of northern Portugal

Submitted



Statement of contribution

The contribution of the candidate, Idalina Bragança, in this work includes the accomplishment of all the experimental work, that is, sample collection and characterization, soils analysis (extraction and quantification), method validation and paper writing.

Assessment of pyrethroid pesticides in topsoils of northern Portugal

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Highlights

- Occurrence of pyrethroid pesticides contaminants in the environment;
- Pyrethroid pesticides assessment in playground parks and agricultural soils;
- The deltamethrin, pyrethroid pesticide, was detected in agricultural soil samples at levels between 15.7 and 101.7 ng g^{-1};
- The results confirm pyrethroid pesticides contamination in Portuguese soils.

Abstract

Pyrethroid pesticides are emerging contaminants broadly used as insecticides for insect pest control in agriculture, veterinary and domestic applications. Limited information can be found in the literature regarding pyrethroid pesticides soils contamination in playground parks and agricultural areas. This study focus on novel findings related to the spatial and seasonal occurrence of pyrethroid pesticides contaminants in soil environment in Portugal. The soils were tested in two seasons, summer and winter. Pyrethroid contamination in measurable levels was not found in the ten playgrounds soil samples analyzed in both seasons. For the eighteen agricultural soil samples tested deltamethrin was the only pyrethroid detected, just in the summer season. Three out of them were found to be positive in concentration between 15.7 and 101.7 ng g^{-1}. These results denote the need of monitoring and assessment of pyrethroid pesticides contamination in Portuguese soils. Further investigation is needed to access the ecological potential impact of pyrethroid pesticides in soil.

Keywords

Soils; pyrethroids, deltamethrin; agriculture, playground parks

1. Introduction

Synthetic pyrethroids (SPs) are chemical pesticides modified from pyrethrins, compounds naturally synthesized by chrysanthemum flowers, with enhanced persistence and toxicity (Palmquist et al. 2012). SPs can be classified in two different types: type I if they are non-cyano pyrethroids or type II if they have the presence of the cyano group. SPs can also be classified as insecticides according to the type of organisms they act against, as they are used for insects pests control in agriculture, public and animal health (Pfeil 2014; S. S. Albaseer et al. 2011). Their wide use are due to being considered between the safer insecticides presently accessible for agricultural and public health purposes (Nicolopoulou-Stamati et al. 2016). The mechanism of SPs action is based on altering the normal function of insect and other invertebrate nervous system by modifying the kinetics of voltage-sensitive sodium channels, leading to knockdown of the normal functioning and eventually to death (Soderlund 2010).

Pyrethroids effects studied in animal models have shown abundant evidence that recognize plausible effects that can be transposed to humans (Burns and Pastoor 2018). Studies that used rats as models suggested that pyrethroids can contribute to the etiology of Parkinson's disease regarding the environmental inductive component (Nasuti et al. 2007), stimulating cognitive deficits (Nasuti et al. 2013). Children are particularly sensitive to environmental contamination with pesticides. They are exceptionally susceptible to uptake and adverse effects of such compounds due to developmental, dietary, and physiologic factors. Studies suggest that exposure to pyrethroid pesticides might be associated with a brain tumors increased risk in childhood (S. Chen et al. 2016) and correlate pyrethroids metabolites with autism spectrum disorders (Domingues et al. 2016).

City parks and agricultural soils are often contaminated with metals (Laidlaw et al. 2018; Ponavic et al. 2018; Y. N. Chen et al. 2018), polycyclic aromatic hydrocarbons

(Ke et al. 2017), polychlorinated biphenyls (Lu and Liu 2015) and pesticides (Li et al. 2008). Few studies were performed is soils of city parks, but in Beijing (China) urban parks soils the organochlorine pesticides hexachlorocyclohexanes (HCHs) and dichlorodiphenyltrichloroethanes (DDTs) were reported at 0.2490–197.0 ng g^{-1} and 5.942–1039 ng g^{-1} concentrations levels, respectively (Li et al. 2008).

More than a dozen registered pyrethroid molecules are used in a numberless of products, depending on the world region (Burns and Pastoor 2018).The intensive use of SPs pesticides raise environmental concerns (Zhang et al. 2011) being their residues frequently detected in soils (Han et al. 2017; Liu et al. 2008), sediments (D. P. Weston et al. 2005; Aznar et al. 2017; Wang et al. 2012; Jabeen et al. 2015) and even in crops (Akoto et al. 2013; Wahid et al. 2017). SPs can be found in worrying concentrations in terms of toxicity for some not-target invertebrates (Donald P. Weston et al. 2013; Palmquist et al. 2012). A major part of the insecticides reaches the soil either by direct application (to kill soil-borne pests) or indirectly when applied to the aerial part of the plant, by runoff from leaves and stems, (Farina et al. 2016). Contaminated soils should be considered an important source of human pyrethroids exposure namely by dermal contact (as children in playgrounds and farmers) or via ingestion by crops cultivated in those soils. Six pyrethroids (bifenthrin, cypermethrin, cyhalothrin, fenvalerate, fenpropathrin and deltamethrin) were found in 65.8% of the investigated nut-planted soils from China, at levels from 1.5 ng g^{-1} to 884.3 ng g^{-1} (Han et al. 2017).

The evaluation of SPs soil contamination should be taken with special consideration where background information on pollution and toxicity should be accessed. Several methods for pyrethroid residues extraction from environmental samples are described in the literature as Soxhlet extraction (Woudneh and Oros 2006), solid-liquid extraction followed by salting-out assisted liquid-liquid extraction (Pastor-Belda et al. 2018), headspace solid-phase microextraction (Fernandez-Alvarez et al. 2008), and microwave-assisted extraction (Esteve-Turrillas et al. 2004). Ultrasound-assisted

extraction is the most common procedure for pyrethroid extraction from soil and sediments (Aznar et al. 2017; Saeed S. Albaseer et al. 2010). Numerous advantages are described for QuEChERS (Quick Easy Cheap Effective Rugged and Safe) method over most of the traditional extraction techniques such as simplicity, low cost, low solvent, and high efficiency with a few number of steps (Vera et al. 2014).

A QuEChERS method with gas chromatography (GC) with electron-capture detector (ECD) was implemented in this work as also described by other authors (Dubey et al. 2018). The optimized method for pyrethroids determination was proven successful being able to quantify trace amounts of eight pyrethroid pesticides (bifenthrin, tetramethrin, cyhalothrin, permethrin, cypermethrin, cyfluthrin, deltamethrin and fenvalerate) in soils. The methodology was proven with ten playground parks soil samples collected in Porto city and eighteen agricultural soils collected in Cávado-Vouga agriculture regional delegation. Both types of soils were evaluated for pyrethroid presence and monitoring in two seasons (summer and winter).

2. Materials and methods

2.1. Chemicals and reagents

For this study, eight of the most widely used SPs were verified: α-cypermethrin, β-cyfluthrin, ʎ-cyhalothrin, bifenthrin, deltamethrin, fenvalerate, permethrin and tetramethrin. Pesticide standards (more than 95.4% of purity) were purchased from Chemservice (West Chester, USA) and Sigma-Aldrich. Merck was the supplier of the acetonitrile and n-hexane (high-purity solvents). Acetonitrile was the solvent used to prepare the individual pesticide standard stock solutions. Standard working solutions were prepared containing the eight studied pyrethroid pesticides in several concentrations levels from stock solutions. Stock and working solutions were stored at 4 °C. Agilent technologies (Bond Elut Sample preparation solutions- Lake Forest, USA) was the supplier of QuEChERS and the dSPE. The QuEChERS used was a buffer-salt mixture with 4 g of magnesium sulphate anhydrous grit ($MgSO_4$), 1 g of sodium

chloride (NaCl), 0.5 g sodium hydrogenocitrate sesquihydrate ($C_6H_8Na_2O_8$) and 1 g of sodium citrate ($Na_3C_6H_5O_7$). The dSPE was composed by 150 mg primary secondary amine (PSA), 150 mg $MgSO_4$ and 50 mg of bonded silica (C18). Deionised water was obtained and purified (18.2 MΩ cm) using water purification systems from Millipore (Elix apparatus and a Simplicity 185 system). Helium (purity ≥ 99.999%) and nitrogen (purity ≥ 99.999%) were both obtained from Linde Sogás.

2.2. Study area and sample collection

Different topsoil samples were collected from several urban playground parks (P1-P10) and agricultural locations (A1-A18) of the north of Portugal. The upper layer (topsoil, 0–10 cm) was removed with a clean spade from each sampling site. Samples were sieved (2 mm) and were stored at -18 °C until analysis.

2.2.1. Playground soils

The sampling was done in urban areas of the north of Portugal in Porto city and surroundings areas playgrounds. Ten soil samples were taken at various locations in Porto city, at 5 and 6 of September 2015 (summer) and 20 and 21 of February 2016 (winter) (Fig. 1).

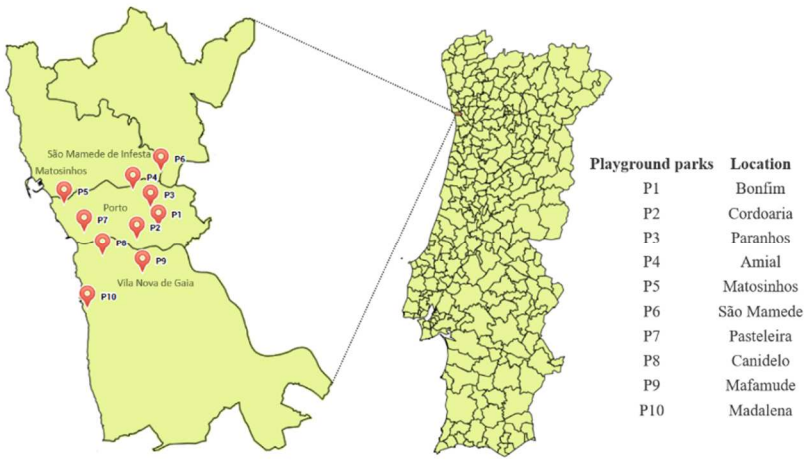
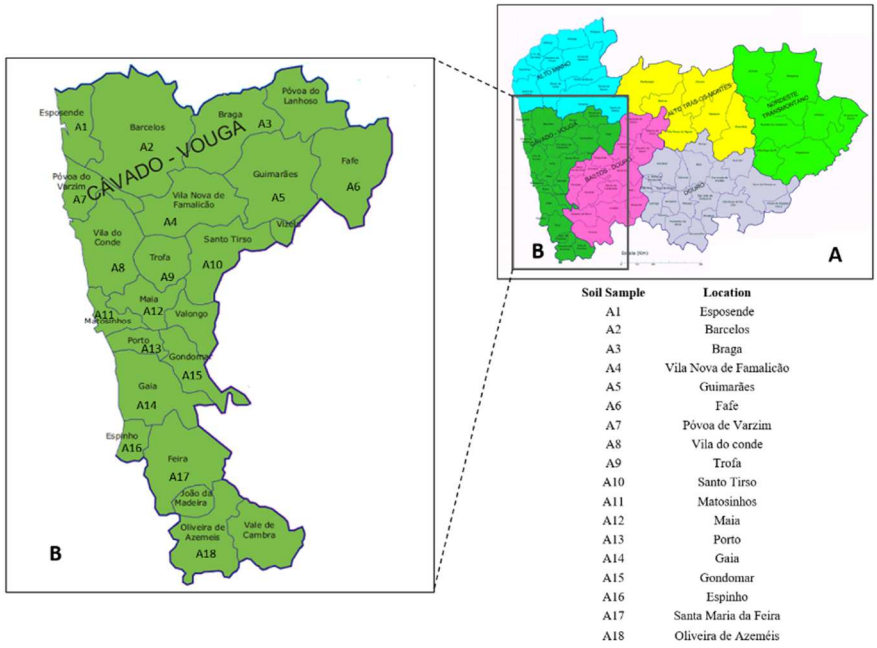


Fig. 1- Location map of playgrounds sampling sites (P1-P10)

2.2.2. Agricultural soils

The agrarian sampling sites were in the agricultural regional delegation of Cávado-Vouga. Eighteen soil samples were collected during the first week of July 2016 (summer) and the first week of January 2017 (winter) at the marked sites (Fig. 2).



Soil Sample	Location
A1	Esposende
A2	Barcelos
A3	Braga
A4	Vila Nova de Famalicão
A5	Guimarães
A6	Fafe
A7	Póvoa de Varzim
A8	Vila do conde
A9	Trofa
A10	Santo Tirso
A11	Matosinhos
A12	Maia
A13	Porto
A14	Gaia
A15	Gondomar
A16	Espinho
A17	Santa Maria da Feira
A18	Oliveira de Azeméis

Fig. 2- Map of agricultural soils sampling locations (A1-A18); A- agriculture regional delegations of the north of Portugal; B- agriculture regional delegation of Cávado-Vouga

Physico-chemical characterization of soils were evaluated namely the pH, the water content and the total organic carbon (TOC)) (Nelson 1996; Hesse 1972). For the determination of TOC in soils a Shimadzu TOC analyzer (VCSN, Japan) and a Shimadzu solid sample module (SSM-5000A) were used. A Kern moisture analyzer (MLS 50-3IR160, Germany) was used to determinate the water content. For measure the pH a mixture (suspension) of soil and water (1:1) was read with an electronic pH meter (Crison 2002, Spain). Triplicate of all the determinations were made.

2.3. Soils analysis

2.3.1. QuEChERS extraction procedure

Soil were extracted using an adapted QuEChERS method from european EN 15662 method (EN15662, 2008). Each sample (5 g) was weighed into a Teflon tube (50 mL) to which 10 mL of acetonitrile were added. The tube was capped, shacked vigorously by vortexing for 1 min and sonicated (10 min) in a 195 W ultrasonic bath from J.P. Selecta (Spain). The QuEChERS buffer-salt mixture was then added to the suspension resultant from this extraction. Phase separation and pesticide partitioning was induced by shacking vigorously for 1 min in vortex and after that was placed in an ultrasonic bath for 10 min and later centrifuged (10 min) at 4000 rpm. A 1.5 mL aliquot was sampled from the upper layer and moved into a 2 mL cleanup tube, vortex for 1 min and centrifuged (10 min) at 4000 rpm. A 0.7 mL aliquot of the upper layer was removed into a vial and evaporated with a nitrogen gentle stream until dryness. The dry residue was resuspended in 0.7 mL of n-hexane, vortex shacked vigorously, and finally the extract was injected into a GC-ECD chromatograph.

2.3.2. Gas chromatography

SPs were analyzed using a GC-ECD (Shimadzu GC-2010) with a capillary column ZB-XLB (30 m length, 0.25 mm i.d., 0.25 µm film thickness, Zebron - Phenomenex). The temperature of the oven was set to start at 60˚C (stay for 1 min), and then increase 30˚C min^{-1} till 250˚C (held for 11 min) and, then an increase of 10˚C min^{-1} till 290˚C. Injection (1 µL) was carry out with a autosampler (HT 300A from hta), the injection port was operated at 250˚C (splitless mode, 2 min) and the detection was done at 300˚C. Helium was used as the carrier gas (1.5 mL min^{-1}), while nitrogen was the makeup gas (30 mL min^{-1}). GC-Solutions Shimadzu was the software used.

In addition, the samples with positive results to deltamethrin were analyzed using a Thermo Trace-Ultra gas chromatography–mass spectrometry Thermo Polaris Q ion trap detector with a column Zebron ZB-5MSi (30 m length, 0.25 mm i.d., 0.25 µm film

thickness -Phenomenex) operated in the electron impact ionization (EI) at 70 eV. Injection (1 µL) was carry out with an autosampler (AI3000) and an injector temperature of 240 ˚C (splitless mode, 0.5 min). Helium was used as carrier gas (1 mL min^{-1}). The temperature of column oven was programmed to start at 90 ˚C (held for 1 min), and then increase 15 ˚C/min to 250 ˚C (held for 1 min), then an increase of 20 ˚C/min to 255 ˚C (held for 5 min), followed increase by 10˚C/min to 270˚C (held for 1 min) and finally increase again 20 ˚C/min to 290 ˚C (stay for 2 min). The transfer line temperature was 270˚C and the ion source temperature was 250 ˚C. The MS/MS conditions were maximum excitation energy 0.225, excitation voltage 0.5 V, excitation time of 15 ms, isolation mass window width 1.0 and isolation time 12 ms. The identification of the selected precursor ion for deltamethrin was 172 as it was the one with the maximum abundance factor and m/z ratio. Xcalibur 1.3 was the software used.

2.4. Method validation

For SPs analysis the experimental method validation was performed according to SANCO guidelines from European Union regarding pesticide residue analytical methods (European commission, 2010, 2013). The matrix effect influence in the ECD signal was evaluated by set-up a calibration curve in soil extract and comparing with the one achieved for the hexane standards. SPs' calibration curves and linear range response were evaluated by analyzing eight standard solutions in the concentration range of 10 to 360 ng g^{-1} in triplicate. It has been described that the matrix effect of the QuEChERS method was affected by the organic carbon content (OC) (Correia-Sa et al. 2012). Two calibration curves were done: one for a soil with a low TOC content (0.374%) and other with high TOC content (3.70%) to ascertain this effect. The detection and quantification limits (LODs and LOQs) were determined using the standard deviation of the response (SD of y-intercepts of regression lines) and the slope of the calibration curve and the signal-to-noise ratio convention, 3:1 for LODs and 10:1 for LOQs (Rambla-Alegre et al. 2012).

The linearity of the method was established by setting calibration curves over the concentration range using linear regression analysis. Selectivity was verified by comparing the chromatograms of standards dissolved in n-hexane, standards extracted from the spiked soil and matrix blanks (non-spiked soil). The precision was evaluated by carrying out the extraction and analysis of 40, 200 and 360 ng g^{-1} fortified samples using three replicates and each extract was injected in triplicate. Analytical method accuracy was evaluated through recovery studies (European commission 2010, 2013).

3. Results and discussion

3.1 Soil sample characterization

The pH values were similar for both type of soils and for both seasons and are registered on the Table 1. Considering water content it was notorious for both type of soils that the water content was lower in summer than in winter which was in accordance with the climatic conditions of each season. Considering that in the winter the rainfall is much higher. The determination of TOC was an important part of soil characterization since its presence/absence could effect how chemicals would respond in the soil (Correia-Sa et al. 2012). TOC contents in agricultural soils (range: 1.24-7.02%) were generally higher than those in playground parks soils (range: 0.03-2.87%).

Table 1 Macro parameters of soil samples. Characterization mean values with standard deviation (SD)

Soil Sample	pH		Water content (%)		Total organic carbon (%)	
	Summer	Winter	Summer	Winter	Summer	Winter
P1	7.20 (0.05)	7.55 (0.06)	1.10 (0.12)	10.11 (0.84)	0.65 (0.04)	0.27 (0.03)
P2	7.16 (0.11)	6.95 (0.09)	9.18 (0.32)	3.86 (0.61)	1.61 (0.20)	0.08 (0.01)
P3	6.31 (0.09)	5.84 (0.01)	12.54 (0.22)	23.99 (0.42)	2.87 (0.39)	2.65 (0.15)
P4	6.31 (0.09)	7.53 (0.02)	4.64 (0.34)	4.23 (0.25)	0.63 (0.06)	0.91 (0.11)
P5	8.52 (0.27)	8.51 (0.07)	3.61 (0.14)	2.88 (0.27)	0.10 (0.01)	0.14 (0.01)
P6	6.87 (0.14)	7.52 (0.11)	1.98 (0.05)	2.45 (0.19)	0.22 (0.01)	0.03 (0.01)
P7	6.33 (0.08)	6.85 (0.03)	0.94 (0.07)	3.03 (0.28)	0.13 (0.03)	0.14 (0.01)
P8	5.70 (0.06)	6.33 (0.08)	8.86 (0.22)	15.93 (0.26)	0.22 (0.01)	0.22 (0.01)
P9	5.59 (0.06)	5.17 (0.18)	5.63 (0.07)	12.35 (1.15)	1.90 (0.25)	1.40 (0.15)
P10	7.80 (0.17)	7.96 (0.16)	5.89 (0.12)	8.35 (0.61)	0.31 (0.03)	0.16 (0.01)
A1	5.65 (0.09)	6.33 (0.04)	14.93 (0.21)	24.98 (0.29)	2.79 (0.05)	2.64 (0.08)
A2	5.71 (0.03)	5.73 (0.10)	13.25 (0.09)	20.35 (0.04)	3.15 (0.24)	2.72 (0.20)
A3	5.51 (0.02)	5.91 (0.02)	16.78 (0.09)	18.83 (0.00)	2.30 (0.26)	2.27 (0.06)
A4	5.39 (0.07)	5.73 (0.01)	19.33 (0.16)	26.33 (0.19)	3.80 (0.08)	2.87 (0.02)
A5	7.03 (0.11)	6.63 (0.08)	9.46 (0.37)	18.24 (0.30)	1.24 (0.06)	1.41 (0.08)
A6	5.42 (0.07)	6.04 (0.13)	17.76 (0.60)	23.45 (0.05)	2.36 (0.04)	1.97 (0.06)
A7	5.86 (0.07)	5.53 (0.03)	14.31 (0.04)	22.93 (0.56)	3.98 (0.34)	3.43 (0.02)
A8	6.97 (0.05)	6.76 (0.12)	21.96 (2.28)	24.51 (0.18)	5.70 (0.08)	5.28 (0.00)
A9	5.91 (0.12)	6.10 (0.16)	9.96 (0.38)	6.82 (0.32)	1.59 (0.10)	2.12 (0.08)
A10	6.91 (0.00)	6.55 (0.15)	9.04 (0.01)	18.94 (0.03)	3.45 (0.51)	2.76 (0.11)
A11	6.68 (0.00)	6.45 (0.04)	19.18 (0.42)	22.20 (0.16)	3.12 (0.11)	3.21 (0.05)
A12	6.76 (0.00)	6.19 (0.01)	15.41 (0.47)	23.14 (0.36)	3.87 (0.23)	3.11 (0.24)
A13	6.64 (0.14)	6.86 (0.12)	19.00 (0.65)	19.96 (0.25)	5.91 (0.55)	5.34 (0.37)
A14	4.52 (0.05)	6.52 (0.09)	17.22 (0.20)	8.03 (0.10)	1.82 (0.13)	2.51 (0.14)
A15	6.72 (0.10)	6.46 (0.01)	25.57 (3.65)	29.48 (0.03)	4.90 (0.12)	7.02 (0.28)
A16	6.96 (0.04)	6.39 (0.01)	2.77 (0.08)	30.03 (0.33)	3.20 (0.11)	3.82 (0.17)
A17	6.77 (0.02)	6.93 (0.08)	1.70 (0.32)	12.95 (0.03)	1.32 (0.04)	2.54 (0.25)
A18	6.59 (0.03)	7.41 (0.04)	17.73 (0.03)	22.67 (0.00)	2.25 (0.27)	2.66 (0.03)

3.2. Method performance and validation

Since some authors reported an influence of the amount of organic carbon in the matrix effect, soils with different TOC contents were tested (Correia-Sa et al. 2012). Comparing the two matrix calibration curves (low and higher TOC content) it was found that a matrix effect to all the compounds, considering different total organic carbon contents, was not observed (data not shown). The calibration curves were constructed with fortified extracts using the soil with low TOC. Chromatograms obtained for extracts with pyrethroid pesticides after the QuEChERS method application are shown in Fig.3 comparing the non-spiked soil (matrix blank) with standards dissolved in n-hexane and also with the spiked soil. The QuEChERS method provide extracts that contained the target analyte, with high recoveries, and free from interferences in the pesticides retention time.

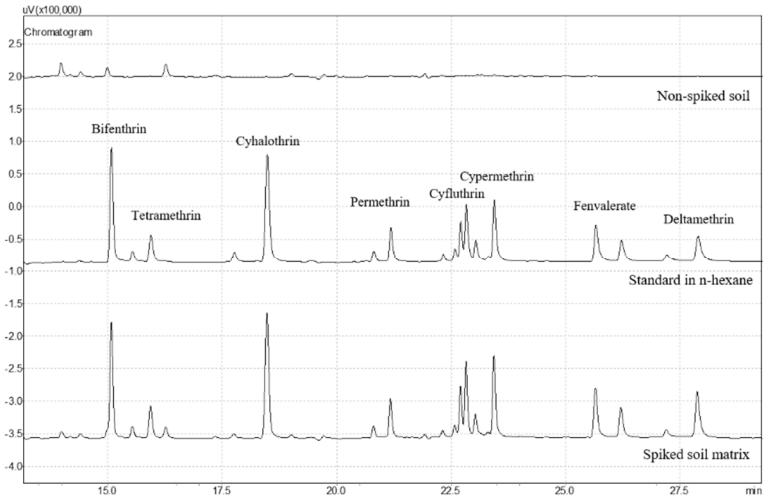


Fig. 3- GC-ECD chromatograms of matrix blanks (non-spiked soil), SPs standards dissolved in n-hexane, SPs in soil spiked matrix.

Analytical parameters for method performance and validation were calculated for the applied method to each pyrethroid pesticide and are reported in Table 2. Method accuracy was presented as recovery from the fortified blank soil samples at 40, 200 and 360 ng g^{-1} concentration levels being good recoveries obtained in the range of

79.4%-100.2% with precision values, determinate by means of relative standard deviation (RSD), below 9.5% in all cases. These determinations comply with the requirements of European Commission guidelines concerning methods to analysis pesticide residue (European commission 2013).

Table 2 Recoveries, detection and quantification limits (LOD, LOQ), and matrix effect for the investigated compounds

Compound	Recoveries, % (RSD, %)			LOD	LOQ	Determination coefficient	Matrix effect
	40 ng g^{-1}	200 ng g^{-1}	360 ng g^{-1}	(ng g^{-1})	(ng g^{-1})	(r^2)	(%)
Bifenthrin	92.8 (6.8)	93.0 (6.5)	80.5 (6.2)	1.73	5.78	0.991	-3.6
Tetramethrin	85.0 (8.4)	92.5 (5.6)	78.8 (6.9)	1.29	4.28	0.991	5.3
Cyhalothrin	79.9 (9.3)	92.2 (6.4)	79.4 (1.2)	2.72	9.06	0.994	8.1
Permethrin	87.6 (9.5)	92.5 (6.7)	81.2 (2.0)	2.34	7.79	0.991	3.5
Cyfluthrin	89.1 (8.5)	99.2 (7.2)	80.4 (4.4)	1.97	6.56	0.997	12.8
Cypermethrin	83.7 (8.0)	95.3 (7.0)	85.3 (3.0)	2.50	8.32	0.997	10.6
Fenvalerate	81.8 (9.1)	95.2 (7.9)	81.2 (2.2)	0.33	1.10	0.998	11.2
Deltamethrin	84.7 (7.8)	100.2 (7.2)	81.2 (1.1)	0.85	2.83	0.997	33.1

Linear calibration curves for all the investigated pesticides were obtained in the range of 10 and 360 ng g^{-1} with coefficients of determination superior than 0.991. Limits of detection and quantification ranged from 0.33 to 2.72 ng g^{-1} and 1.10 ng g^{-1} and 9.06 ng g^{-1}, respectively.

3.3. Pyrethroid in soils

Eight pyrethroid pesticides (i.e. bifenthrin, deltamethrin, cyhalothrin, cyfluthrin, cypermethrin, fenvalerate, permethrin, and tetramethrin) were analyzed in playground parks and agricultural soils. When the validated procedure for the monitoring of pyrethroid pesticide residues was applied to ten non-spiked soils samples from Portuguese Porto city playgrounds no pyrethroid contamination was found in significant levels (not detected) in samples analyzed in both seasons.

Considering the pyrethroids effects by dermal contact (Hołyńska-Iwan et al. 2018) these city playgrounds may be considered safe for the children. However, the importance of future analyses in a wider territory, with higher number of samples and including more pyrethroid compounds is of major importance since these urban facilities are frequently visited, and an understanding of the environmental quality and the impact of these compounds in daily life is crucial.

Table 3 contains the results of the pyrethroids contamination detected in agricultural soils. Deltamethrin was the only pyrethroid pesticide confirmed by GC-MS/MS, of the eight analyzed. It was detected in three of the eighteen agricultural soils only in the summer period, while in winter no pyrethroid contamination was found. The occurrence only happening in summer may be related to their usage pattern, being the pesticides usually applied in the spring/summer, as advices by the Ministry of Agriculture Forestry and Rural Development (DGAV).

Table 3 Concentrations (ng g^{-1}) of Deltamethrin in north of Portugal agricultural soils.

Soil Sample	Deltamethrin (ng g^{-1})	
	Summer	Winter
A1	n.d	n.d
A2	n.d	n.d
A3	n.d	n.d
A4	n.d	n.d
A5	101.7 ± 7.2	n.d
A6	n.d	n.d
A7	n.d	n.d
A8	17.8 ± 0.2	n.d
A9	n.d	n.d
A10	n.d	n.d
A11	n.d	n.d
A12	n.d	n.d
A13	n.d	n.d
A14	n.d	n.d
A15	n.d	n.d
A16	n.d	n.d
A17	n.d	n.d
A18	15.7 ± 0.7	n.d

Deltamethrin as many pyrethroids suffer microorganism's biodegradation since they have several esterase enzymes capable of degrading pyrethroids so they are not detected in winter. When pyrethroids were present in measurable amounts, their concentrations were 15.7, 17.8 and 101.7 ng g^{-1} for samples A18, A8 and A5, respectively. Comparing with other places in the world, in China, six SPs (bifenthrin, fenpropathrin, cyhalothrin, cypermethrin, fenvalerate and deltamethrin) were found in soil samples, with the concentrations of 29.4 ng g^{-1}- 884.3 ng g^{-1}, 9.7 ng g^{-1}- 57.3 ng g^{-1} and 1.5 ng g^{-1}- 85.0 ng g^{-1}, for chestnut, walnut and pinenut soils, respectively (Han et al. 2017). Sediments of creeks near Roseville (USA) inside a residential neighborhood presented maximum concentrations of 169, 335, 437 and 736 µg kg^{-1} of cyfluthrin, permethrin, bifenthrin, and cypermethrin, respectively (D. P. Weston et al. 2005). SPs (bifenthrin, cyhalothrin, cyfluthrin, cypermethrin, deltamethrin, fenpropathrin, fluvalinate, permethrin, esfenvalerate and resmethrin) were also detected in Mediterranean paddy fields at a maximum concentrations of 57.0 ng g^{-1} before plow and 62.3 ng g^{-1} during rice production. Resmethrin and cyfluthrin were the compounds detected at higher concentrations (Aznar et al. 2017). Deltamethrin were also found in other studies reported in the literature. For instance, in chestnut soil in China (Han et al. 2017) deltamethrin varied between n.d to 29.3 ng g^{-1} (Han et al. 2017). Considering crops, nine pyrethroids (bifenthrin, fenopropathrin, cyhalothrin, permethrin, cyfluthrin, cypermethrin, fenvalerate, allethrin and deltamethrin) were measure in all cowpea and maize samples with mean concentrations in cowpea ranged from 1 to 39 ng g^{-1} while that in maize was from 2 to 28 ng g^{-1} from Ejura (Ghana). The ranging levels of deltamethrin detected were between 2 - 3 ng g^{-1} and 3 - 13 ng g^{-1} in maize and cowpea, respectively (Akoto et al. 2013).

The pyrethroids levels found in the analyzed agricultural soils were higher than those reported to cause sediment toxicity in California's Central Valley, taking into account other pyrethroid (bifenthrin) that reached a maximum concentration of 32 ng g^{-1} concentration higher than the LC50 of 3 - 10 ng g^{-1} for some non-target invertebrates (*Hyalella azteca* and *Chironomus dilutus*), even when sampling was not definitely focused on areas of great pyrethroids use (Donald P. Weston et al. 2013).

4. Conclusions

The intensive use of pyrethroid pesticides raise environmental concerns, especially regarding soil contamination. The spatial and seasonal occurrence of pyrethroid pesticides contaminants in the environment was the focus of this work. The methodology developed for pyrethroids analysis could be extremely relevant to further studies related with pyrethroids exposition and effects. We found no evidence that in Portuguese playground soil samples the pyrethroid pesticides analyzed would be present, considering that nothing was found in the ten urban samples collected in the northern region of the country. Nevertheless, 17% of the agricultural soil samples tested had measurable levels for one target pyrethroid (deltamethrin) in the summer season. The results confirm pyrethroid pesticides contamination in Portuguese soils and the need of monitoring and assessment. In order to circumvent this problem tests should be done in a wider territory, with a higher number of samples and include more pyrethroid compounds, both in urban and rural facilities. A deeper apprehension of the environmental quality and the ecological potential impact of pyrethroid pesticides in soil is needed.

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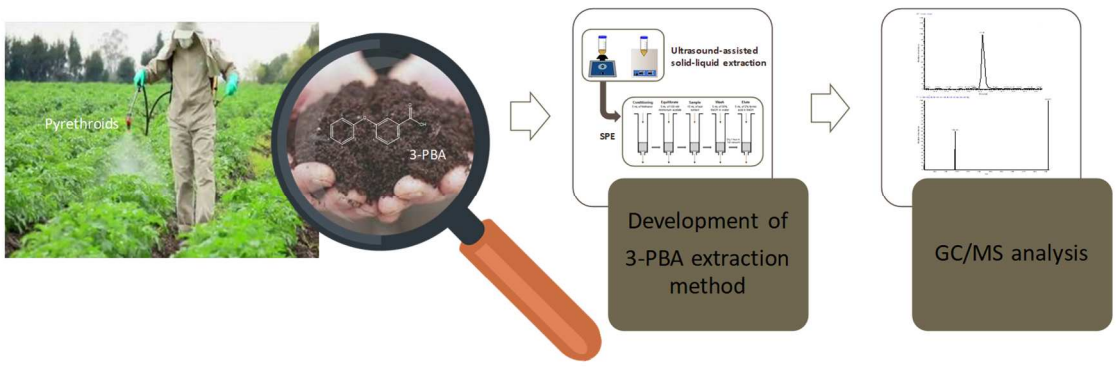
2.2 Pyrethroid pesticide metabolite, 3-PBA, in soils: method development and application to real agricultural soils

Submitted



Statement of contribution

The contribution of the candidate, Idalina Bragança, in this work includes the accomplishment of all the experimental work, that is, sample collection and characterization, method development, soils analysis (extraction and quantification), method validation and paper writing.

Pyrethroid pesticide metabolite, 3-PBA, in soils: method development and application to real agricultural soils

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Highlights

- An analytical method that combines an aqueous solid-liquid extraction, the SPE procedure, and GC/MS detection has been introduced for determination of 3-PBA in soil samples for the first time;
- An SPE method at ng/g level has been validated for the detection of 3-PBA in soil;
- Recoveries at the three fortification levels ranged from 70.3 and 93.5%;
- The pyrethroid metabolite, 3-PBA, was detected in agricultural soil sample at levels of few nanograms per gram.

Abstract

3-Phenoxybenzoic acid (3-PBA) is a shared metabolite of several synthetic pyrethroid pesticides (SPs) resulting from environmental degradation of parent compounds and thus occurs frequently as a residue in samples. Hence, the importance of 3-PBA evaluation after pyrethroids application. There is a gap of analytical methods to determine 3-PBA in soil samples. Therefore, an analytical method that combines the solid phase extraction (SPE) and gas chromatography/mass spectrometry (GC/MS) detection have been developed for the determination of 3-PBA in soil samples. The analytical method was validated in terms of linearity, sensitivity, intra- and inter-day batch precisions, recoveries and quantification limits. An SPE method using a Strata X cartridge allows obtaining limits of detection and quantification equal to 4.0 and 13.3 ng g^{-1}, respectively. Under optimized conditions the method average recoveries levels ranged from 70.3 and 93.5% with a relative standard deviation below 3.4%. Method intra- and inter-day precision was under 5.0 and 4.8%, respectively. The developed method was applied to eleven agricultural soil samples in the north of Portugal. The developed methodology allowed for the determination of the pyrethroid metabolite, 3-PBA, in agricultural soil samples at levels of few ng g^{-1}.

Keywords

3-phenoxybenzoic acid; solid phase extraction, soils, Gas chromatography/mass spectrometry

1 Introduction

Synthetic pyrethroids (SPs) are a class of pesticides commonly used around the world as insecticides. These pesticides are derived from pyrethrins, which are natural insecticides produced by certain species of chrysanthemum flowers (Palmquist et al. 2012). Pyrethroids vary from many other pesticides as they have extreme hydrophobicity, rich stereochemistry (contain one to three chiral centers) and broad-spectrum high-level insecticidal activity. These pyrethroids represent a significant enhancement when compared to other insecticides classes as a result of their low non-target toxicity and high selectivity to target species (Luo and Zhang 2011). There are several registered pyrethroid molecules that are used in a myriad of products for agriculture, veterinary, domestic and medical applications (Burns and Pastoor 2018).

A review that summarizes the available studies (between 1986 and 2017) focus on pyrethroid residues in different media at the global scale indicated that pyrethroids have been widely detected in a range of environmental compartments (including soils (Fernandez-Alvarez et al. 2008a) (Regueiro et al. 2007; Yao et al. 2010), water (Kumari et al. 2008; Li and Chen 2013), sediments (Amweg et al. 2006; Feo et al. 2010), and indoors (Leng et al. 2005; Yoshida 2009)) and in organisms (Corcellas et al. 2015; Kittusamy et al. 2014). In this review, the presence of pyrethroids metabolites was only reported for biological samples including human urine and other excretions (Tang et al. 2018).

3-Phenoxybenzoic acid (3-PBA) is a metabolite of several synthetic pyrethroid pesticides and occurs by degradation of parent compounds. 3-PBA has a pka of 3.92, is water soluble (24.7 mg L^{-1}) and its octanol-water partition coefficient (Log P) is 3.91 (Pesticide Properties DataBase). 3-PBA is not a specific biomarker of exposure to a particular pyrethroid, because it results from environmental degradation and it's a shared metabolite of a number of commonly-used pyrethroid pesticides (Aylward et al. 2018), as can be seen in Figure 1 (Chen et al. 2011a; Kaneko 2010; Liang et al. 2005; Maloney et al. 1988; Tallur et al. 2008). Research on the toxic effects of SPs metabolites is still limited, however, they could induce multiple toxic responses like parent compounds, and their toxicity should be considered for improving the understanding of environmental risks of SPs (Xu et al. 2018). A review study, regarding data from 15 published articles from observational exposure of children to pyrethroids, reported 3-PBA as the most frequently detected pyrethroids exposures biomarker (Morgan 2012). This metabolite has shown stronger reproductive toxicity, weaker hydrophobicity, and a longer half-life than the parent compounds. Consequently, this metabolite is more likely to accumulate in the environment, causing secondary pollution

of agricultural products (Meyer et al. 2013; Vidal et al. 2009; White et al. 1996).

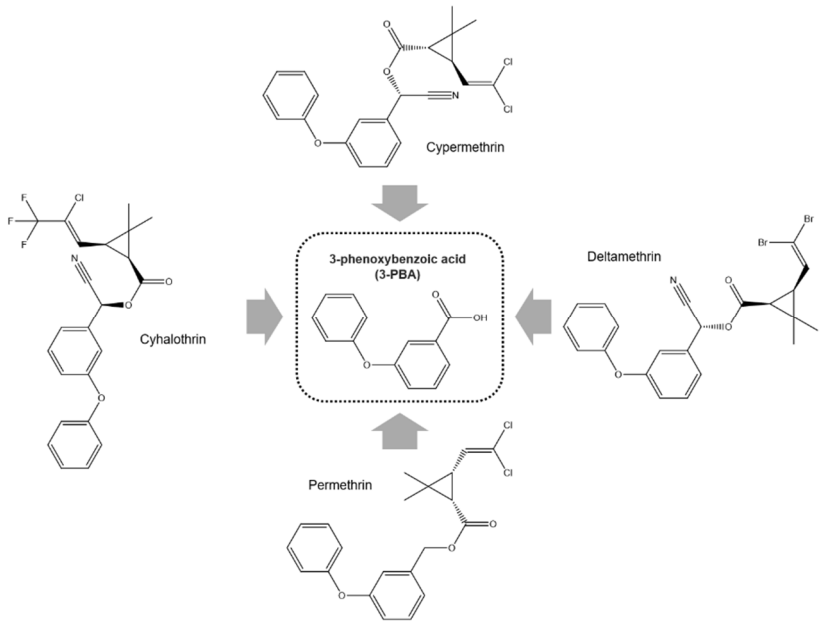

Fig. 1 The structures of some pyrethroids (cyhalothrin, cypermethrin, deltamethrin, and permethrin) and the mutual and major metabolite, 3-phenoxybenzoic acid

There is an ongoing interest in the potential associations of 3-PBA exposure in individuals as an effective way to ensure the safety of food, the living environment, and occupational exposure levels (Ueyama et al. 2010). The 3-PBA is persistent and refractory to degradation in natural environment with half-life in soil is reported ranging from 120 to 180 days (Chen et al. 2011b; Halden et al. 1999). Additionally, 3-PBA can enter the aqueous phase, but it tends to be absorbed to the soil/sediment (Chen et al. 2012). The importance of developing a method for pyrethroid metabolite determination in soils is not only related to their accumulation in soils or organisms but also with the constant application of pyrethroid insecticides (Ortiz-Hernández et al. 2013) and the rule of this metabolite as pyrethroids contamination indicator.

Currently several analytical methods are described in the literature for the quantification of 3-PBA, however, these reports are focused on 3-PBA presence in urine samples (Fedeli et al. 2017; Jain 2016; Schettgen et al. 2016; Ye et al. 2017). Enzyme-linked immunosorbent assay (ELISA) methods can be employed for the determination of 3-PBA in urine (Ahn et al. 2011; Chuang et al. 2011; Matveeva et al. 2001; Shan et al. 2004). Within the analytical methods used there are two mainly extraction methods applied, i.e. liquid-liquid extraction (LLE) and solid phase extraction (SPE). For this purpose, SPE is the most widely used preconcentration procedure since it is used not only to extract traces of organic compounds from environmental samples but can also

remove interfering components from the matrix (Domingues et al. 2016; Rodriguez-Mozaz et al. 2007). Although SPE has a higher cost and more washing steps, this method is better than LLE in terms of higher selectivity, easier handling, and hazardous solvents reduction. QuEChERS (Quick Easy Cheap Effective Rugged and Safe) is also a reported technique for pesticide extraction as it has some advantages comparing with traditional extraction techniques such as simplicity, low cost, low solvent, and high efficiency (Vera et al. 2014). Additionally, detection methods such as liquid chromatography-tandem mass spectrometry (LC-MS/MS) or gas chromatography/mass spectrometry (GC/MS) were the most described (Arrebola et al. 1999; Columé et al. 2001; Ueyama et al. 2010). Derivatization procedure is necessary prior to gas chromatography/mass spectrometry pyrethroid metabolite detection.

There is a gap of analytical methods to determine 3-PBA in soil samples. Therefore, the aim of this work was to develop a sensitive analytical method to determine 3-PBA in soils and apply it to real samples. Preliminary studies were done testing two solid phase extraction methods: Acetonitrile (ACN) solid-liquid extraction and QuEChERS method. The developed analytical method combines an aqueous solid-liquid extraction with the SPE procedure by using a Strata X cartridge and GC/MS detection. This procedure was successfully applied to eleven agricultural soil samples in the north of Portugal and to the best of our knowledge, it was the first time that the pyrethroid metabolite, 3-PBA, was analysed in agricultural soil samples at levels of few ng g^{-1}. This methodology has the potential to simplify unbiased monitoring of 3-PBA in soil samples and to access contamination outcomes.

2 Materials and methods

2.1 Reagents, solvents, and materials

3-phenoxybenzoic acid (98%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade solvents, such as n-hexane, ACN, methanol and ethyl acetate were purchased from Merck (Darmstadt, Germany). HPLC-grade formic acid (99%) and ammonium acetate (>98%) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

The derivatization reagents 1,1,1,3,3,3-Hexafluoro-2-propanol (HIPF, ≥99.8%) and N,N'-Diisopropylcarbodiimide (DIC, 99%) were from Sigma-Aldrich (St. Louis, MO, USA). The QuEChERS and the dispersive solid phase extraction (dSPE) clean-up were supplied by Agilent technologies (Bond Elut Sample preparation solutions) (Lake Forest, CA, USA). QuEChERS is a buffer-salt mixture consisting of 4 g of magnesium sulphate anhydrous grit, 1 g of sodium chloride, 0.5 g sodium hydrogenocitrate sesquihydrate and 1 g of sodium citrate. The dSPE was composed by 150 mg

magnesium sulphate, 150 mg primary secondary amine (PSA), and 50 mg C18. Ultra-pure water was prepared using a Milli-Q water purification system (Millipore, Billerica, MA, USA). SPE columns containing different sorbents were purchased from Phenomenex (Torrance, CA, USA) (Table 1). The stock standard solution of 3-PBA (at a concentration of 242 mg L^{-1}) was prepared on a weight basis by dissolving the standard compound in ACN and were stored in a refrigerator at 4 °C.

2.2 Soil sampling and characterization

The sampling sites were located in the north of Portugal in the regional delegation of agriculture of Cávado-Vouga. Eleven different agricultural soil samples were collected during July 2016, represented in Figure 2. At each sampling site, the upper layer (0–10 cm) was collected with a spade. Soils were sieved to a grain size of 2 mm to obtain a homogeneous sample, before being extracted and analysed, and were stored at -18 °C until analysis. Macro parameters, such as water content, total organic carbon content (TOC) and pH, were evaluated (Hesse 1972; Nelson 1996). For the determination of TOC in soils a Shimadzu TOC analyzer (model VCSN, Shimadzu, Japan) with a solid sample module (SSM-5000A) was used. Water content was determined using a moisture analyzer (Kern MLS 50-3IR160, Germany). For measure the pH a mixture (suspension) of soil and water (1:1) was read with an electronic pH meter (Crison 2002, Spain). Triplicate of all the determinations were made.

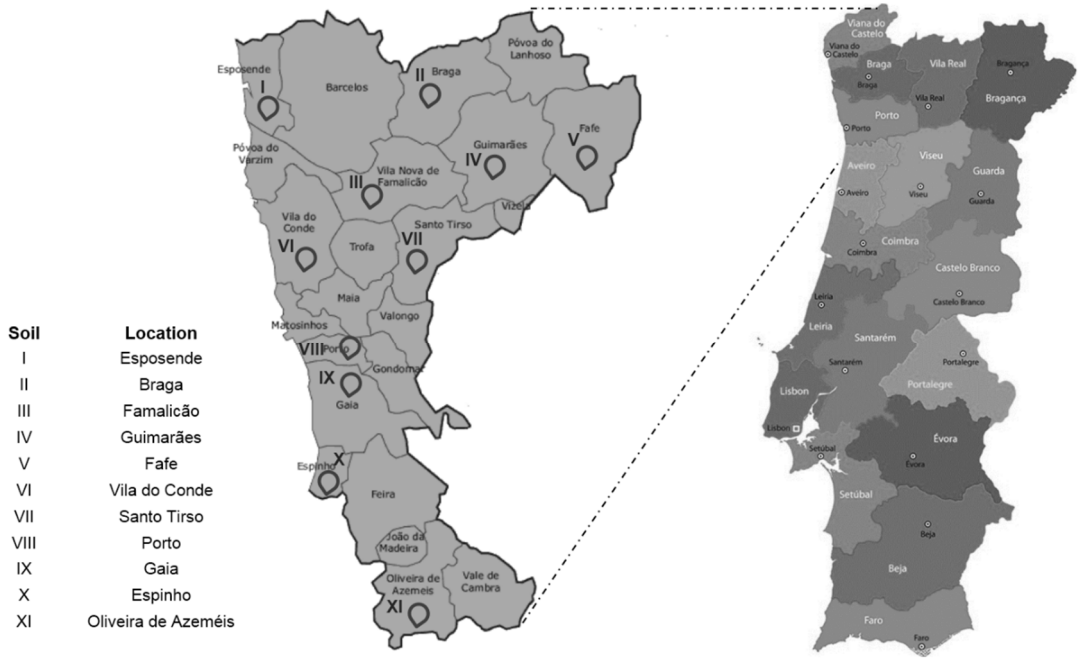

Fig. 2 Geographical location of the soil samples

Table 1
Comparation of the SPE cartridges based on composition, packing (sorbent amount/cartridge volume), particle and pore size, and application.

Cartridge	Composition	Sorbent amount/ cartridge volume	Particle size (µm)	Pore size (Å)	Application
Strata-C18-E	Octadecyl modified silica	200 mg/6 mL	55	70	Nonpolar compounds
Strata-X	PS-DVB-VP	200 mg/3 mL	33	85	Acidic, basic and neutral compounds
Strata-X-A	PS-DVB modified with amine groups	30 mg/3 mL	33	85	Acids
Strata-X-C	PS-DVB modified with sulfonic groups	200 mg/3 mL	33	85	Bases

Table 2
Solid phase extraction procedures tested for the different cartridges.

Procedure	Cartridge	Conditioning	Equilibrate	Load	Wash	Dry	Elute
I	Strata-C18-E	methanol	water	3-PBA in water	30% methanol in water	1 hour (full vacuum)	5% methanol in ethyl acetate
II	Strata-X	ACN	water		30% methanol in water		acetonitrile
III		methanol	water		30% methanol in water		2% formic acid in methanol
IV	Strata-X-A	ACN	water		ammonium acetate 100mM methanol		5% formic acid in methanol
V		methanol	water		ammonium acetate 100mM methanol		5% formic acid in ACN
VI	Strata-X-C	ethyl acetate methanol	water HCl 0.1N	3-PBA in water (pH adjusted to 2.5)	HCl 0.1N NH_4OH		5% methanol in ethyl acetate

2.3 Sample preparation

Several procedures of sample preparation were tested to evaluate 3-PBA and all the tests were spiked with an intermediate 3-PBA standard solution of 20000 µg L^{-1} in ACN. The spiking amounts added were calculated to have in the final extract a 100 µg L^{-1} of 3-PBA concentration, and the spiking solvent was evaporated with a gentle stream of nitrogen.

2.3.1 QuEChERS method

Soil samples, spiked with 3-PBA, were extracted using a QuEChERS method adapted from Yang et al. (2010). The amounts of this method were reduced to half, which was described as follows: each sample (5 g) was weighed into a 50-mL centrifuge tube, then 10 mL of ACN was added to the tube. After capping the tube was vortex shaken vigorously for 1 min and after that, the tubes were placed for 10 min in an ultrasonic bath. A QuEChERS buffer-salt mixture was added to the suspension derived from the first extraction to induce phase separation and pesticide partitioning. The closed tube was shaken vigorously by vortex for 1 min, then the tubes were sonicated for 10 min in an ultrasonic bath and were centrifuged for 5 min at 4500 rpm. The ultrasonic bath was used as a homogenization technique as it was described before to improve the obtained results, as soil is a complex and heterogeneous matrix (Braganca et al. 2012; Vera et al. 2013).

For both methods described in preliminary studies, after centrifugation, the extracts were subjected to a clean-up step. So, an aliquot of 1.5 mL was sampled from the upper layer and transferred into a 2 mL dSPE clean-up tube and vortex for 1 min and then centrifuged for 5 min at 8000 rpm. An aliquot of 0.5 mL from the upper layer was transferred into a vial and evaporated to dryness with a gentle stream of nitrogen. These dry residues were then subjected to derivatization process.

2.3.2 Optimization of an extraction process combining aqueous solid-liquid extraction followed by SPE

To select the most appropriate SPE extraction process, 15 mL of 3-PBA water solution were processed via solid phase extraction using four different copolymer sorbents in Phenomenex^{R} cartridge format: Strata-C18-E, Strata^{TM}-X-A, Strata^{TM}-X-C and Strata^{TM}-X. Table 1 presents a summary of the characteristics of each SPE cartridge. Methods adapted from those suggested by SPE cartridges suppliers (Table 2) were used to evaluate the cartridge performance to 3-PBA extraction.

For aqueous soil extraction, a 10 g of soil sample was added to a 50 mL Teflon centrifuge tube, and extraction with 30 mL of different aqueous solvents (water or the

buffer ammonium acetate 100 mM) were evaluated. The mixture was vortexed for 1 min, ultrasonicated for 10 min in a 195 W ultrasonic bath from J.P. Selecta (Spain) at room temperature, and centrifuged at 4500 rpm for 5 min. Then, 15 mL of the upper layer of the soil extract was passed through SPE cartridges.

For the SPE cartridge that allowed for the best recoveries, a study was performed by optimizing different ratios (1:6, 1:3 and 1:2) of mass of soil sample (g) per volume (mL) of extraction solvent, using 5, 10 and 15 g of soil for 30 mL of extraction solvent. The SPE was preconditioned with 5 mL of methanol and 5 mL of water, for obtaining the best conditions in extraction of the analyte from the soil sample. The precondition step of equilibration of the cartridge described in table 2, was also done with ammonium acetate instead of water, followed by methanol conditioning. The cartridge was washed with 5 mL of mixed solvent (methanol/water, 30/50, v/v) and finally eluted by 5 mL of 2% formic acid in methanol. An aliquot of 0.5 mL was taken and dried in a gentle stream of nitrogen. This dry residue was then subjected to the derivatization process.

2.4 Derivatization

Derivatization procedure was necessary prior to gas GC/MS analysis. 3-PBA derivatization of the dry residues from the extraction methods tested above was performed by the addition of 30 µL HFIP and 20 µL of DIC to the previously described dry residues prevenient from the extraction procedures and slightly shaken (vortex, 1600 rpm) for 10 min, at room temperature. In the final phase of the procedure, a LLE was performed with 1 mL of a 5% aqueous potassium carbonate solution (to neutralize the excess derivatizing agent) and 500 µL of n-hexane in the vial with 5 min vortex (1600 rpm) shake. An aliquot of the organic layer (200 µL) was transferred to the autosampler vials for GC/MS analysis.

2.5 Gas chromatography analysis

A volume of 1 µL was injected onto a Thermo Trace-Ultra gas chromatograph, coupled to an ion trap mass detector Thermo Polaris, operated in the electron impact ionization at 70 eV. The ion source temperature and the MS transfer temperature were at 250ºC. Operating in the splitless mode (0.5 min), the helium was used as carrier gas at a constant flow rate of 1.3 mL min^{-1}. The injector was maintained at 240ºC. The column, a 30 m ZB-5MSi (0.25 mm i.d., 0.25 µm film thickness Zebron-Phenomenex), oven temperature was programmed as follows: initial temperature 40˚C (held for 1 min), increased by 15˚C/min to 160˚C (held for 0.5 min), increased by 15˚C/min to 180˚C (held for 1 min) and finally increased by 20˚C/min to 250˚C. A program was developed

in the SIM mode, based on the detection of selected ions for 3-PBA (141, 196 and 364).

2.6 Method validation

For 3-PBA analysis, the experimental method validation was performed according to the European Union SANCO guidelines on pesticide residue analytical methods (European commission 2010; 2013). The influence of the soil matrix in the GC/MS signal was evaluated by preparing a n-hexane and a match-matrix 3-PBA calibrations curves. To assess the matrix effect (ME), the slope of the match matrix calibration curve was compared with the slope of the calibration curve prepared in hexane. The calibration curves and linear ranges of the detector response for 3-PBA were evaluated by analysing the working standard solutions (15–180 µg L^{-1}, 8 concentrations) in triplicate. In this study, the LOD and LOQ were calculated as the minimum amount of analyte detectable with a signal-to-noise ratio (S/N) of 3 and 10, respectively. The linearity of the method was established by setting calibration curves using linear regression analysis over the concentration range. Selectivity was verified by comparing the chromatograms of the standards dissolved in n-hexane, the standards extracted from the spiked soil and the matrix blanks (non-spiked soil). The accuracy of the analytical optimized method was evaluated through recovery studies at three concentration fortification levels (low: 90, medium: 600 and high: 1080 ng g^{-1}), using three replicates. The intraday precision and the inter-day precision of the method were evaluated at 600 ng g^{-1}, the intermediate concentration of the spiking level. The intraday precision of the assay was estimated by calculating the relative standard deviation (RSD) for the analysis of soil samples in six replicates on one day (n=6). Inter-day precision was determined by the analysis of three replicates of soil samples on three consecutive days (n=9).

3 Results and discussion

3.1 QuEChERS extraction

The extraction of 3-PBA with ACN ultrasound-assisted solid-liquid extraction even with QuEChERS extraction procedure showed to be inefficient, as the results obtained were below the n-hexane calibration curve limit of detection (LOD= 0.69 µg L^{-1}). It was found that the extraction with acetonitrile for this compound is not a good choice as it was not even possible to calculate recoveries. Therefore, considering the solubility of 3-PBA, an aqueous ultrasound-assisted solid-liquid extraction follow by SPE proved to be a promising 3-PBA extraction technique.

3.2 Aqueous solid-liquid extraction follow by SPE

3.2.1 Selection of SPE cartridge and method

Previous assays were performed using aqueous solutions of 3-PBA to optimize the SPE extraction step. A total of four different SPE cartridges were tested for a concentration of 100 µg L^{-1} of 3-PBA prepared in water. For SPE, the protocols were adapted from the Phenomenex® recommended protocols. The recovery results (%) are shown in Figure 3 for triplicate replicates.

By analysing the recoveries of the various SPE columns it is possible to verify that those that are within the limits recommended for pesticides residues analysis in the range of 70 to 120% with RSD ≤20 (Albaiges 2016) are the procedure III with Strata X cartridge and the procedure IV with Strata X-A cartridge (see table 2). These results are in accordance with the properties of 3-PBA (acid compound, pka of 3.92) Thus, these cartridges/procedures were chosen to optimize 3-PBA SPE extraction for the soil samples.

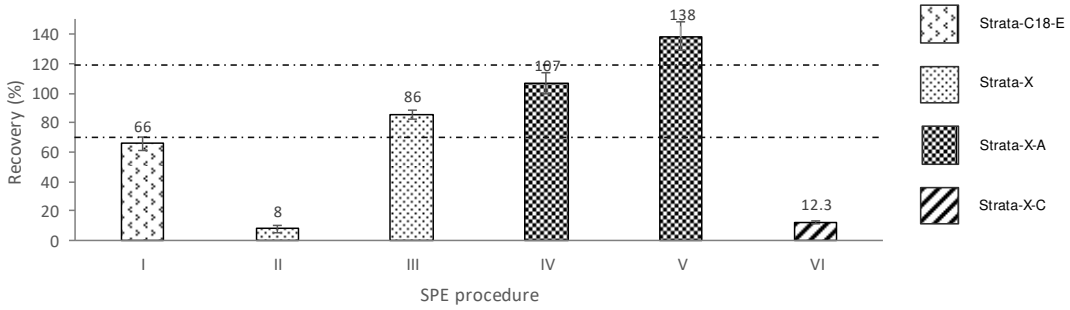

Fig. 3 Recoveries of 3-PBA extraction in water using different SPE cartridge. I to VI are the different solid phase extraction procedures tested for the different cartridges

3.2.2 Optimization of soil extraction

Comparing the recoveries of the same amount of 3-PBA adding in soil and adding in water, it was obtained lower results in soils. Because, probably, the 3-PBA extraction from soil was not efficient. So, an optimization of the soil extraction procedure was required. The effect of using the buffer, ammonium acetate 100 mM as extraction solvent instead of water was evaluated for both cartridges (Figure 4, a) and the best recovery was found for the cartridge Strata X with ammonium acetate as the soil extraction solvent (49.2 ± 4.3%). The influence of the amount of soil (Figure 4, b)) was also tested (5, 10 and 15 g soil).

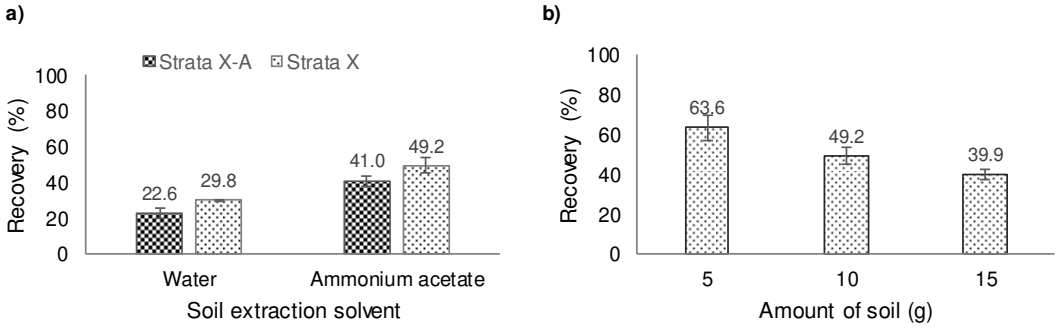

Fig. 4 Optimization of soil extraction by changing: a) extraction solvents b) ratio of mass of sample per volume of solvent

The recovery values ranging between 39.9% for 15 g to 63.6% for 5 g of soil. It is notorious that the last one (5 g) allows better results. To improve the SPE process after the conditioning step with methanol, the cartridge was equilibrated with the same solution used in soil extraction i.e. ammonium acetate 100 mM. This change in SPE equilibration step allowed an improvement in the recovery from 63.6 ± 2.6% when using water to a value of 72.2 ± 1.1% when the ammonium solution was used. As ammonium acetate is used as the extraction solvent, it is also the best solvent to be used to equilibrate the SPE cartridge, as was proved by the enhancement of 3-PBA recoveries. The optimized process is shown in Figure 5.

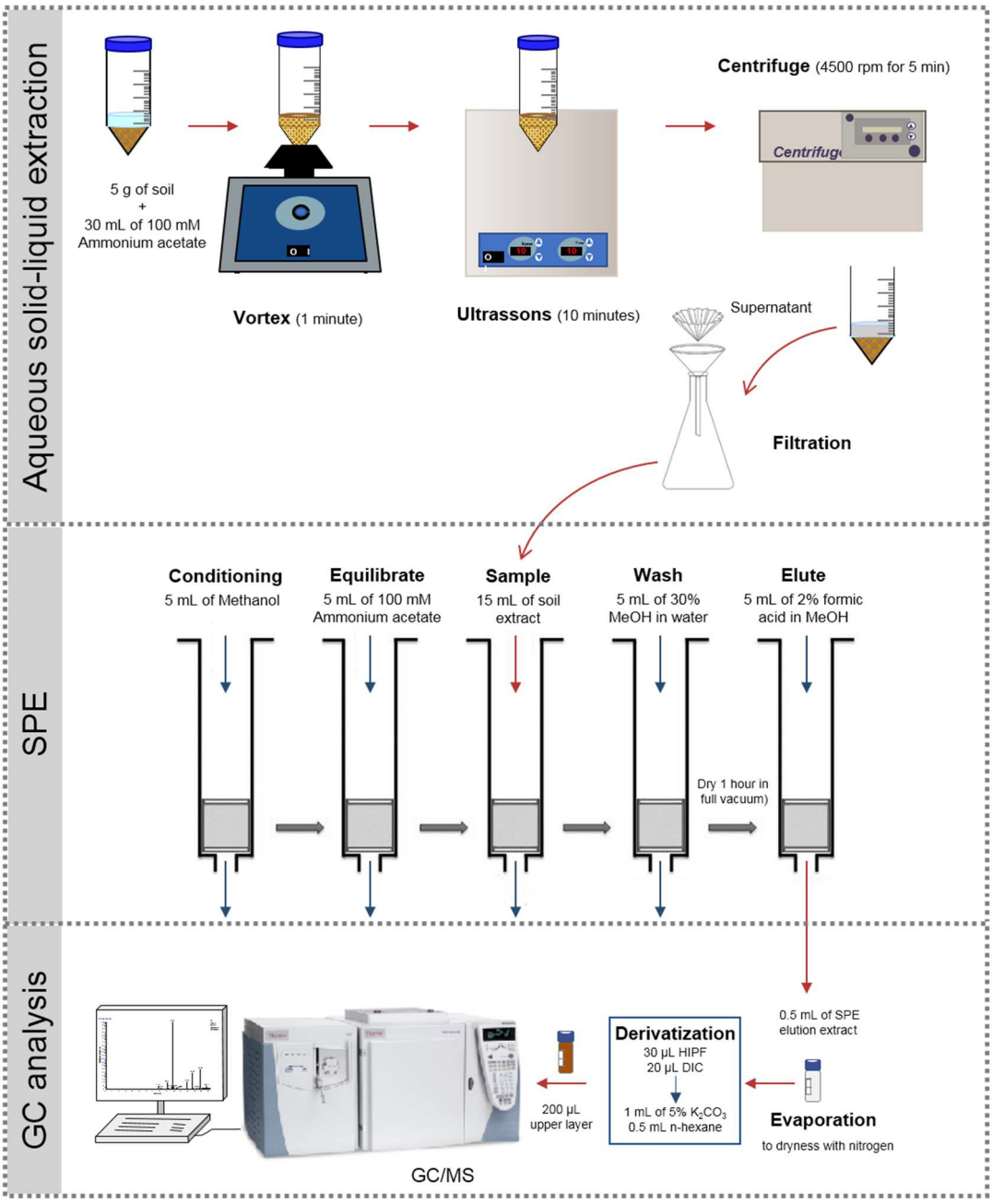

Fig. 5 Scheme of the optimized procedure for 3-PBA determination in soil

3.2.3 Method performance

A complete method validation comprising linearity, selectivity, sensitivity, accuracy, precision, and ruggedness was performed. In addition, matrix effect was studied. An organic agricultural soil with 3.70% of TOC content and a pH of 6.91 was use for method validation. At 3-PBA retention time (11.9 min), no interferences from endogenous substances were detected. Consequently, a good separation was obtained under the described GC/MS conditions. A chromatogram of a soil sample spiked with 100 µg L^{-1} of 3-PBA is depicted in Figure 6.

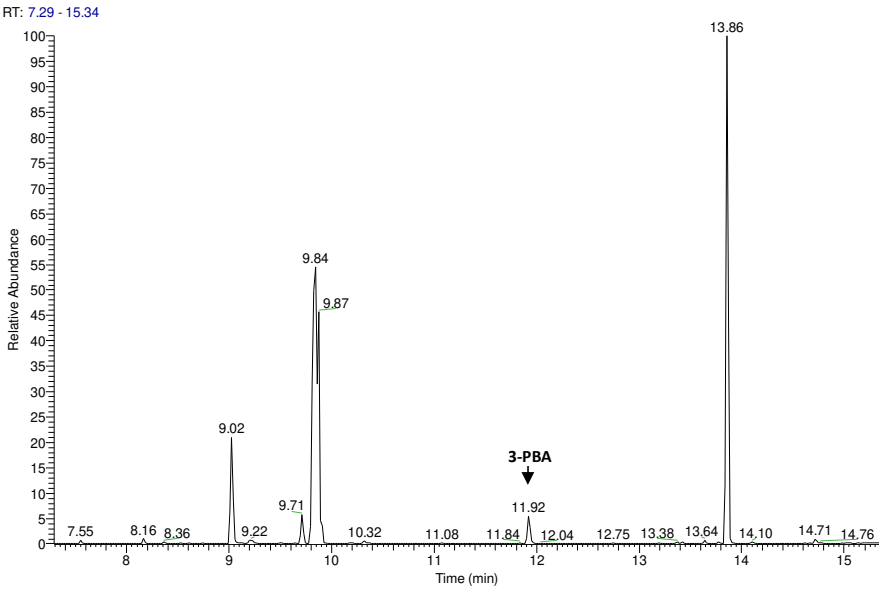

Fig. 6 Gas chromatography/mass spectrometry under selected ion mode chromatogram of 3-PBA at a spiking level of 100 µg L^{-1} in the soil sample

The ME (%) was obtained from the ratio of linear relationships from the slopes in reagent-only and in match-matrix calibrations and is equal to 91.0% (<100% ionization suppression) which represents a low ionization suppression (less than 10%). Therefore, for soil samples, match matrix calibrations curve was necessary for this method to improve its accuracy. A good linear relationship was obtained between the response and their corresponding concentrations (90-1080 ng g^{-1}) with an R^2 = 0.9989. The obtained LOD and LOQ were of 4.0 and 13.3 ng g^{-1}, respectively.

Validation of both accuracy and precision of the optimized method were obtained for three spiking levels (90, 600 and 1080 ng g^{-1}, n = 3 for each spiking level) and the results are shown in Figure 7.

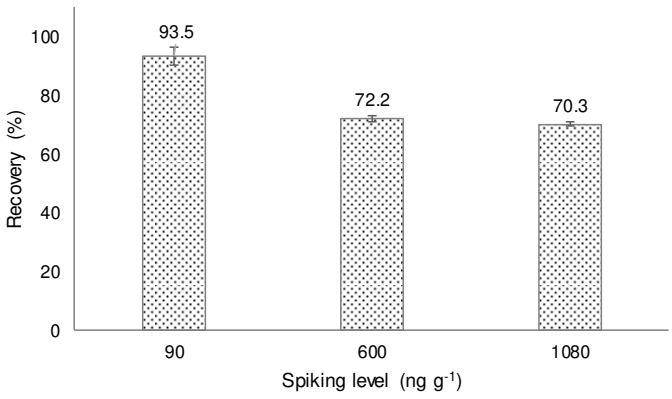

Fig. 7 Recoveries obtained with extraction at three spiking level concentrations (90, 600 and 1080 ng g^{-1}) (n = 3)

Overall, the recoveries ranged from 70.3 to 93.5% and RSDs ranged from 1.1 to 3.4%. The precision of the method was assessed in terms of repeatability (intraday) and reproducibility (inter-day) for 3-PBA at the medium spiking level concentration (600 ng g^{-1}) and the results were equal to 5.0% and 4.8%, respectively. The results showed that the proposed method could be used for effective monitoring of 3-PBA in soil samples.

4. Application to real samples

The developed and optimized method was applied for the determination of 3-PBA in eleven different agricultural soils. The samples were collected in July 2016. The pH values were similar for all soils and are registered on the Table 3. The determination of TOC was an important part of soil characterization since its presence or absence could influence how chemicals would react in the soil (Correia-Sa et al. 2012). The contents of TOC in agricultural soils range between 1.24 and 5.91%. Only one soil was found to be positive for 3-PBA contamination in real samples. corresponding to soil IV with 23.2 ± 1.7 ng g^{-1}. To confirm this, result more extractions of this soil were done and concentrated 5 times, the final extract was quantified in the linear range of the matrix calibration curve obtaining the same results. As far as the authors know no references were found reporting the presence of 3-PBA in soil samples.

Table 3 Soil samples characterization, mean values with standard deviation (SD)

Soil	pH	Water content (%)	Total organic carbon (%)
I	5.65 (0.09)	14.93 (0.21)	2.79 (0.05)
II	5.51 (0.02)	16.78 (0.09)	2.30 (0.26)
III	5.39 (0.07)	19.33 (0.16)	3.80 (0.08)
IV	7.03 (0.11)	9.46 (0.37)	1.24 (0.06)
V	5.42 (0.07)	17.76 (0.60)	2.36 (0.04)
VI	6.97 (0.05)	21.96 (2.28)	5.70 (0.08)
VII	6.91 (0.00)	9.04 (0.01)	3.45 (0.51)
VIII	6.64 (0.14)	19.00 (0.65)	5.91 (0.55)
IX	4.52 (0.05)	17.22 (0.20)	1.82 (0.13)
X	6.96 (0.04)	2.77 (0.08)	3.20 (0.11)
XI	6.59 (0.03)	17.73 (0.03)	2.25 (0.27)

This metabolite is most frequently detected pyrethroid biomarker (>67%) has it can enter the human body in various ways (food, the residential environment, soil and various environmental media containing pyrethroids (Tang et al. 2018). Few reports were found worldwide regarding the presence of the parent compounds (pyrethroid pesticides) in soils due to affinity to organic carbon (Domingues et al. 2007). Most reports were found on the Asian continent, mostly from China followed closely by India. These results could be explained by the high usage pattern of insecticides in China, which has resulted in serious pesticide pollution. The highest concentration found were in Chongqing cropland (906.05 ng g^{-1}) (Tang et al. 2018). In Europe, a study in Spain using a developed headspace solid-phase microextraction detected several pyrethroid pesticides, at concentrations below the generic reference levels established by Spanish legislation in soils (Fernandez-Alvarez et al. 2008b). 3-PBA, the pyrethroid metabolite was previously only detected in human body residues worldwide (Tang et al. 2018). Nothing was found regarding its presence in soil environmental samples nor even methods to determine the metabolites of pyrethroid pesticides in soils.

Conclusions

A new, simple, rapid and robust analytical method for the determination of pyrethroid metabolite, 3-PBA, in soils was developed based on aqueous solid-liquid extraction with the buffer, ammonium acetate followed by SPE procedure and GC/MS detection. The detection and quantification limits in the low ng g^{-1} range (4.0 and 13.3 ng g^{-1}, respectively) were achieved. The method average recoveries levels ranged from 70.3 and 93.5% with a relative standard deviation below 3.4%. Intra- and inter-day precision was under 5.0 and 4.8%, respectively. Good validation parameters such as accuracy, precision, and linearity proved suitability for purpose of the developed method.

The developed method was successfully applied to the analysis of eleven agricultural soils, showing the occurrence of 3-PBA, at levels up to few ng g^{-1} of soil (~23 ng g^{-1}). The presence of pyrethroid major metabolite in agricultural soils points out the relevance of extending monitoring programmes to the analysis of these compound as well as the parent compounds.

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CHAPTER 3

Deltamethrin impact in a cabbage planted soil

Deltamethrin impact in a cabbage planted soil: degradation and effect on microbial community structure

Submitted

Statement of contribution

The contribution of the candidate, Idalina Bragança, in this work includes the sample collection, deltamethrin and 3-PBA determination (extraction, detection and method validation), DNA extraction, data analysis and paper writing.

Deltamethrin impact in a cabbage planted soil: degradation and effect on microbial community structure

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Highlights

- Monitoring of deltamethrin on the applied cabbage and soils over time;
- NGS as a tool to understand how microbial communities responded to deltamethrin application;
- Natural microbial community potentially biodegrades deltamethrin and 3-PBA;
- Shifts in soil microbial community structure after 30 days of pesticide application.

Abstract

Synthetic pyrethroids (SPs) are one of the most common pesticides used worldwide. Their use has greatly increased in the last decades and its' continuous application lead to increased pesticides concentration in soil that, consequently may enter the food chain affecting environment and human health. The monitoring over time of deltamethrin degradation, a pyrethroid pesticide, was carried out following their evolution on the applied cabbage and soils. Its progression was correlated with the changes in the natural microbial community, which may include microorganisms with potential to biodegrade deltamethrin and its metabolite, 3-phenoxybenzoic acid (3-PBA). Next-generation sequencing of the 16S rRNA gene amplicon was employed to understand how microbial communities responded to the deltamethrin application. Shifts in soil microbial community structure, were observed after 30 days of pesticide application. The main changes were the increased abundance of *Nocardioides* sp. and *Sphingomonas* sp., associated with deltamethrin and 3-PBA consumption, respectively. Although deltamethrin was not found in any of the tested samples (soil and cabbage) after 180 days, it caused an environmental impact much further than the 7 days security interval described in this insecticide product label. These findings suggest that deltamethrin application can disturb soil microbial community and that natural biodegradation can be an important element for pesticides soil decontamination.

Keywords

Deltamethrin; 3-phenoxybenzoic acid; soil; microbial community; next-generation sequencing

1. Introduction

External agricultural inputs such as pesticides, organic amendments and fertilizers are applied to maximize productivity, whereas side effects on soil microorganisms are frequently neglected. Pesticides, in general, allowed a remarkable increase in agricultural yield and food production (Aktar et al., 2009). They became an important factor to worldwide agricultural systems as they enable that agricultural production follows the growing trend of world population increase (Carvalho, 2017). In 2017 the United Nations estimated that by 2100 the world population will reach 11.2 billion (2017). Synthetic pyrethroids (SPs) are one of the most common pesticides species used (Burns and Pastoor, 2018), and its utilization greatly increased in the last decades due to their effectiveness and low toxicity when compared to other insecticides (Yoo et al., 2016). Although pesticides are extremely important for pest control, their application can cause effects to human health and to the environment (Tang et al., 2018). Its constant application lead to increased concentrations of pesticides in soil and that consequently could enter the food chain (Akoto et al., 2013; Ortiz-Hernández et al., 2013; Han et al., 2017). According to the European Commission the good agriculture practices presume that pesticides should be applied so that the highest level of a pesticide residue found on food or feed is the legally tolerated (maximum residue level (MRL)) (EU, 2005). The main source of pesticides contamination is the direct application to agricultural crops although some accidental spills from manufacturing or animal ectoparasites control can also occur (Ortiz-Hernández et al., 2013; Farina et al., 2016). Due to their high hydrophobicity SPs can strongly bind to soil and organic matter and can be toxic to non-target organisms in the environment (Cycoń and Piotrowska-Seget, 2016).

Some literature reviews have addressed the impact of pesticides and others agriculture inputs on soil microorganisms. Regarding pesticides, few effects have been documented for herbicides, while for fungicides and insecticides negative effects are common (Bünemann et al., 2006). The impact of soil contaminants can be assessed by analysing the soil microbial community changes, in their composition, number and activity. Changes in the equilibrium of soil microbial community can be a first indicator for negative alterations in the soil conditions (Donkova and Kaloyanova, 2008). Microbial community can be characterized in terms of functional diversity and structure by using environmental deoxyribonucleic acid (DNA) sequence data and related metadata, across natural or experimental conditions (Larsen et al., 2012). Many studies have investigated methods for DNA extraction, purification, amplification, and quantification from soils (Tsai and Olson, 1991; Zhou et al., 1996; Miller et al., 1999;

Martin-Laurent et al., 2001). DNA extraction is a crucial step as the extraction protocol may influence the outcome of a microbial community structure (Zielińska et al., 2017).

Microbial community structures of environmental samples can be determinate by means of amplicon next-generation sequencing (NGS) over the use of specific gene markers such as species-specific DNA metabarcoding (Shokralla et al., 2012). This technique allows a better sample diversity representation and an increase in data acquisition at a reasonable cost which makes metagenomic sequencing technologies very attractive for DNA analysis in ecological and environmental research (TABERLET et al., 2012; Soliman et al., 2017). Sequence-specific separation of partial 16S rDNA amplification were used to access the impact on microbial soil communities with the herbicides dinoterb (Herbogil) and metamitron (Goltix) as model compounds. Important pattern variation was found with different and extremely reproducible community patterns resulting from different herbicides application (Engelen et al., 1998). The impact of the application of methylpyrimifos and chlorpyrifos on an agricultural soil was assessed by following the soil microbial community. These insecticides were used at normal pest control level and it was found a significant decreased in aerobic nitrogen-fixing bacteria and consequently in nitrogen fixation (Martinez-Toledo et al., 1992). For the organophosphate insecticide methyl parathion its long-term effect in the diversity of soil microbial community was studied by using small subunit ribosomal RNA gene-based cloning. Analyzing the sequences of the dominant phylotypes, authors noticed that the bacterial communities changed markedly. The dominant bacterial groups present in control soil (bacillus genus, and a member of α-proteobacteria) were replaced in contaminated soil by a member of the flexibactera-cytophaga-bacteroides division and two members of the γ-proteobacteria (*Pseudomonas* sp.) (Zhang et al., 2006).

The environment of the contaminated site plays a crucial role in the success of pollutant detoxification, particularly the natural microbial community present as they can be the first responsible for pesticide degradation in soil (Zouboulis and Moussas, 2011). SPs and even their metabolites can be degraded by different microorganisms (Bragança et al., 2016). The importance of microbial metabolism in the degradation or detoxification of SPs residues in the environment has been demonstrated by Maloney et al. (1988). Many microorganisms were reported to degrading SPs such as *Acidomonas* sp (Paingankar et al., 2005), *Azoarcus indigens*, (Ma et al., 2013), *Ochrobactrum lupin* (Chen et al., 2011a), *Sphingobium* sp. (Guo et al., 2009), *Pseudomonas aeruginosa* (Zhang et al., 2011) *Bacillus cereus* and *Streptomyces aureus* (Chen et al., 2012b). 3-Phenoxybenzoic acid (3-PBA) occurs by SPs environmental degradation, being a shared metabolite of several commonly-used

pyrethroid pesticides (Maloney et al., 1988; Liang et al., 2005; Tallur et al., 2008; Kaneko, 2010; Chen et al., 2011b; Aylward et al., 2018). The biodegradation process can be crucial to accomplish pesticide environment decontamination (Singh, 2002).

The present study aimed to investigate the biodegradation in soil of an applied commercial formulation of a pyrethroid pesticide (deltamethrin). The study was conducted to research the potential impact of the pyrethroid applied at normal pest control level. The monitoring of deltamethrin degradation in cabbage and soils over 180 days was conducted and the microbial community dynamic was monitored in order to assess the impact of deltamethrin application on the natural community and to identify some of the bacteria that could potentially be involved in the biodegradation of deltamethrin and its metabolite, 3-PBA.

2. Materials and methods

2.1 Chemicals and reagents

Deltamethrin (99%) and 3-phenoxybenzoic acid (98%) standards were obtained from Chemservice and Sigma-Aldrich, respectively. The pyrethroid pesticide Decis® EC 25 (25 g L^{-1} of deltamethrin) was obtained from Bayer CropScience. HPLC-grade formic acid (99%) and ammonium acetate (>98%) were obtained from Sigma-Aldrich. Merck was the supplier of the high-purity solvents n-hexane and acetonitrile (ACN). Individual deltamethrin and 3-PBA standard stock solutions were prepared in ACN. All stock and working solutions were stored at 4 °C. For deltamethrin soil extraction a QuEChERS method was used consisting of a buffer-salt mixture of 4 g of magnesium sulphate anhydrous grit ($MgSO_4$), 1 g of sodium chloride (NaCl), 0.5 g sodium hydrogenocitrate sesquihydrate ($C_6H_8Na_2O_8$) and 1 g of sodium citrate ($Na_3C_6H_5O_7$). An AOAC dispersive solid phase extraction (dSPE) was used as clean-up with the composition of 50 mg primary secondary amine (PSA), 150 mg $MgSO_4$ and 50 mg of bonded silica (C18). For cabbage deltamethrin extraction a QuEChERS method AOAC (6 g $MgSO_4$ + 1.5 g sodium acetate) followed by a clean-up AOAC with a pigment dSPE clean-up (50 mg PSA + 50 mg Bulk carbongraph + 50 mg C18(EC) + 150 mg $MgSO_4$) were used. Agilent technologies was the supplier of QuEChERS and the dSPE. Strata X cartridge was purchased from Phenomenex. The derivatization reagents 1,1,1,3,3,3-Hexafluoro-2-propanol (HIPF, ≥99.8%) and N,N'-Diisopropylcarbodiimide (DIC, 99%) were from Sigma-Aldrich. Deionised water was produced and purified (18.2 MΩ cm) using an Elix apparatus and a Simplicity 185 system, both equipment from Millipore. PowerSoil DNA Isolation Kit was supplied from MO BIO Laboratories.

2.2 Insecticide application

The application of Decis® EC 25 in the agricultural soil was done by spray drift at a normal pest control level in the field. The concentration used by the farmer was 75 mg L^{-1} of deltamethrin active ingredient (3 mL of deltamethrin-commercial formulation per 1 L of water). The Decis® EC 25 concentration used was the recommended from the supplier for field application (sale, distribution and application of phytopharmaceuticals are regulated by law number 26/2013 (DGAV, 2013). This commercial formulation has a security interval for cabbage of 7 days after application as described in Bayer CropScience product flyer.

2.3 Sample collection

The agricultural sampling site was located in Guimarães city (north of Portugal, about 65 km from Porto city). The agricultural soil was planted with collard greens (*Brassica oleracea* sp.) commonly known as Portuguese cabbage. Soil sampling started to be collected by the end of June 2016 and finished in early January 2017. The first sample was taken previous to pesticide application (G0) and the other samples were collected after 1, 3, 7, 15, 30 and 180 days of application (G1, G3, G7, G15, G30 and G180, respectively). At each sampling time the soil upper layer (0–10 cm, topsoil) was collected with a clean spade. For DNA analysis, soil samples were collected aseptically and frozen at -80 °C, until processing. For deltamethrin and 3-PBA analysis, soil samples were sieved (2 mm) and stored at -18 °C. Two cabbage leaves were collected each sampling time, were cut into small pieces with a knife and then frozen in a freezer bag at -80 °C. Before being used for deltamethrin extraction the frozen cabbage was crushed inside the freezer bag taking advantage of the fact that it was at -80 ºC which simplified the milling process.

2.4 Analytical determinations

2.4.1 Deltamethrin and its metabolite determination

A short description of the analytical procedures used for the determination of deltamethrin and 3-PBA is presented in Table 1. Briefly, deltamethrin was extracted by using the QuEChERS methods EN 15662 (2008) and AOAC (2007) for soil and cabbage, respectively. 3-PBA extraction method combines an ammonium acetate (100 mM) solid-liquid extraction with the SPE procedure by using a Strata X cartridge. For deltamethrin and 3-PBA determination the European Union SANCO guidelines were followed for method validation (European commission, 2010, 2013).

A first order model $C_t=C_0e^{-kt}$ was used as described before for the biodegradation kinetics of the SP (Chen et al., 2011a; 2011b; 2011c), where k is the rate constant (day^{-1}), C_0 is the amount of deltamethrin at first time after application (time 1) and Ct is the amount of deltamethrin after t time (30 days). The $T_{1/2}$ was determined by $T_{1/2}=ln2/k$.

Table 1

Brief description of the analytical procedures used for the determination of deltamethrin (soil and cabbage) and 3-PBA (soil).

Compound	Sample	Extraction	Detection
Deltamethrin	Soil	QuEChERS EN15662 (2008) dSPE clean-up	Quantification: Shimadzu GC-2010 with an ECD detector, equipped with capillary column of 30 m, ZB-XLB (0.25 mm i.d., 0.25 µm film thickness, Zebron - Phenomenex) Confirmation: Thermo Trace-Ultra gas chromatography–mass spectrometry Thermo Polaris Q ion trap detector with a column ZB-5MSi (30 m length, 0.25 mm i.d., 0.25 µm film thickness Zebron-Phenomenex). The identification selected precursor ion for deltamethrin was 172.
	Cabbage	QuEChERS AOAC (2007) dSPE clean-up (AOAC + pigment)	
3-PBA	Soil	Ammonium acetate (100 mM) solid-liquid extraction SPE procedure with a Strata X cartridge	Thermo Trace-Ultra gas chromatography–mass spectrometry Thermo Polaris Q ion trap detector with a column ZB-5MSi (30 m length, 0.25 mm i.d., 0.25 µm film thickness Zebron-Phenomenex) A program was developed in the SIM mode, based on the detection of selected ions for 3-PBA (141, 196 and 364).

2.5 Characterization of microbial community

2.5.1 DNA extraction

DNA extraction was performed in soils (250 µg of homogenized samples) using the PowerSoil DNA Isolation Kit (MO BIO Laboratories). DNA quality was evaluated through visualization on 1.5% agarose gels and each DNA preparation was quantified with the Qubit fluorometer (Invitrogen).

2.5.2 Next generation sequencing

Microbial communities from soils were characterized by next generation sequencing. Samples were prepared for Illumina Sequencing by 16S rDNA gene amplification of the prokaryotic community. The samples were sent to Genoinseq (Cantanhede, Portugal) facilities for analysis. Briefly, the DNA was amplified for the hypervariable V4-V5 region of the 16S rDNA with specific primers. First polymerase chain reactions (PCR) were performed for each sample using KAPA HiFi HotStart PCR Kit according to manufacturer recommendation with 0.3 µM of each PCR primer (forward 515F-Y (5'-GTGYCAGCMGCCGCGGTAA -3') and reverse 926R (5'-CCGYCAATTYMTTTRAGTTT -3') (Parada et al., 2016) and 12.5 ng of template DNA in a total volume of 25 µL. The PCR conditions involved: a 3 min denaturation at 95 ºC, followed by 25 cycles of 98 ºC for 20 s, 50 ºC for 30 s and 72 ºC for 30 s and finally 5 min at 72 ºC. The second PCR reactions added indexes and sequencing adapters to both ends of the amplified target region according to manufacturer's recommendations (Illumina, 2013). Negative PCR controls were included for all amplification procedures. The amplified products obtained were purified and normalized with SequalPrep 96-well plate kit (ThermoFisher Scientific, USA) (Comeau et al., 2017), pooled and pair-end sequenced in the Illumina MiSeq® sequencer with the V3 chemistry, according to manufacturer's instructions (Illumina, USA) at Genoinseq. Raw reads were extracted from Illumina MiSeq® System in fastq format and quality-filtered with PRINSEQ version 0.20.4 (Schmieder and Edwards, 2011) to remove sequencing adapters, reads with less than 100 bases and trim bases with an average quality lower than Q25 in a window of 5 bases. The forward and reverse reads were merged by overlapping paired-end reads with AdapterRemoval version 2.1.5 (Schubert et al., 2016) using default parameters.

2.5.3 Data analysis

Merged reads were uploaded and processed for downstream analysis by the automatic pipeline Silva Next Generation Sequencing (SILVAngs 1.3) (http://www.arb-silva.de/) (Quast et al., 2013). Microbial composition of each soil sample, at different taxonomic level, was determinate using pipeline's default setting. Specifically, reads were aligned with the SILVA Incremental Aligner (SINA v 1.2.10 for ARB SVN (revision 21008)) (Pruesse et al., 2012) against the SILVA SSU rRNA SEED and checked according to the following quality filtering parameters: max ambiguity 2%, max homopolymers 2%; min seq. quality 30%, min length 200 bp, min align. identity 50%. Remaining reads were dereplicated and clustered at 98% of similarity using cd-hit-est (Li and Godzik, 2006) to generate Operational Taxonomic Units (OTUs); the classification was done against SILVA SSU Ref dataset release 132 using blastn version 2.2.30+ with standard settings (Camacho et al., 2009). Reads without any BLAST hits or reads with weak BLAST hits (%sequence identity + % alignment coverage/2 < 93), remained unclassified and were labelled as "no relative". Rarefaction curves were extrapolated by calculating 100 data points (Quast et al., 2013).

Statistical analyses were performed with the software packages PRIMER 6 (version 6.1.11) (Clarke and Gorley, 2006). Microbial community dynamic was evaluated through a hierarchical cluster analysis, by normalizing the matrix using presence/absence pre-treatment function and Bray–Curtis similarity method. Clusters were generated using group average method and SIMPROF test was performed to test differences among generated clusters.

3. Results and discussion

3.1 Method performance and validation

Parameters as recovery, detection and quantification limits (LOD, LOQ), and linearity were calculated for the analytical methods used for deltamethrin and 3-PBA determination and are described in Table 2. The accuracy of the method was expressed as recovery from fortified blank samples at three concentration levels of 15/20 (3-PBA/Deltamethrin), 100 and 180 µg L^{-1} and good recoveries were obtained in the range of 79.4%-100.2% with relative standard deviation (RSD) values below 9.5% in all cases. These results comply with the requirements of the European Commission concerning pesticide residue analytical methods (EU 2013).

3.2 Deltamethrin and 3-PBA degradation

After the application of the deltamethrin commercial formulation, both cabbage and soil samples were monitored over time for the presence of deltamethrin (Fig. 1). Before the application (time 0) deltamethrin was not detected in soil and the same result was obtained after 180 days of application. The highest concentrations were found for cabbage. For days 1 and 30 after application the deltamethrin content ranged from 829 to 64 ng g^{-1} for cabbage and from 600 to 56 ng g^{-1} for soil, respectively. The degradation half-life time ($T_{1/2}$), the time in which the compound concentration is reduced by 50%, was calculated for deltamethrin in soil. The $T_{1/2}$ was determined and was equal to 8.8 days and the degradation constant k was 0.079 day^{-1}. In field conditions pyrethroids degraded in aerobic soils with highly variable half-lives ranging from 3 to 96 days (Laskowski, 2002). Similar results were reported by Chen et al. (2011) with an isolated bacteria from activated sludge, *Ochrobactrum lupin* DG-S-01, that was capable to degraded deltamethrin with a half-life of 8.2 days and a rate constant of 0.084 day^{-1}.

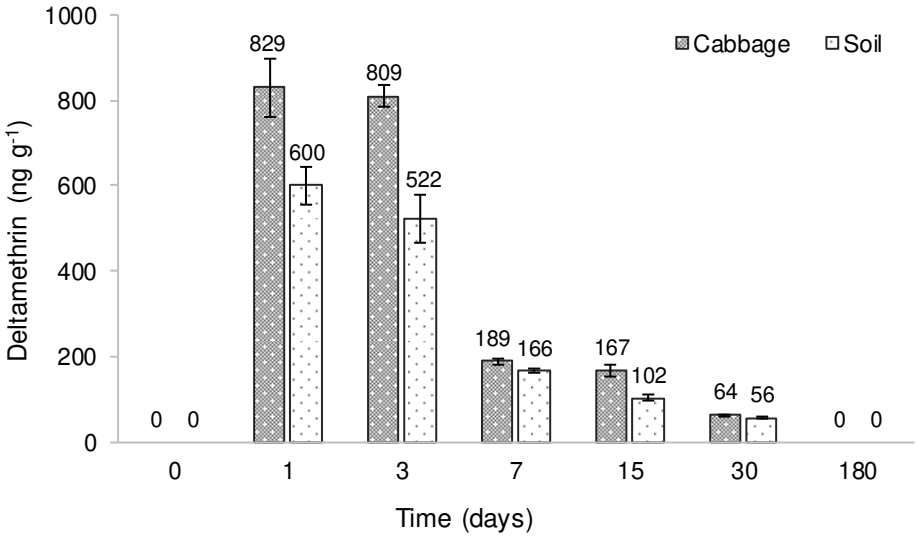

Fig. 1. Deltamethrin evolution in soils and cabbage samples over time after Decis® EC 25 application.

Table 2

Recoveries, detection and quantification limits (LOD, LOQ), and linearity for deltamethrin and 3-PBA

Compound	Sample	Linear Range ($\mu g\ L^{-1}$)	Spiking levels ($\mu g\ L^{-1}$) Recoveries, % (RSD, %)			R^2	LOD ($ng\ g^{-1}$)	LOQ ($ng\ g^{-1}$)
			20	**100**	**180**			
Deltamethrin	Soil	5-180	84.7 (7.8)	100.2 (7.2)	81.2 (1.1)	0.997	0.85	2.83
	Cabbage	5-180	95.6 (5.4)	83.7 (6.8)	79.7 (1.7)	0.994	1.44	4.79
			15	**100**	**180**			
3-PBA	Soil	15-180	93.5 (3.2)	72.2 (1.1)	70.3 (0.8)	0.999	4.0	13.3

Regarding the deltamethrin concentrations in cabbage, the European union pesticides database established the maximum residue levels for leafy *Brassica* between 0.01 and 0.2 mg kg^{-1} (European commission, 2005). Collard greens cabbage is a specie of leafy *Brassica* not present in this database so "other" maximum residue level that corresponds to the lower limit of analytical determination (0.01 mg kg^{-1} i.e. 10 ng g^{-1}) must be consider. The deltamethrin commercial formulation applied had a security interval for cabbage of 7 days. It is notorious that cabbages presented deltamethrin concentrations higher than the maximum residues levels after the 7 days of security interval and even after 30 days of the pesticide application.

To better understand the deltamethrin degradation in soil, the concentration of its metabolite, 3-PBA, was assessed (Fig. 2). Together with the deltamethrin decrease it was possible to see that the metabolite 3-PBA increased over time accomplishing the maximum value (23.2 ng g^{-1}) after 15 days of deltamethrin application. Considering, the absence of 3-PBA in day 0, the surge of this compound in the following days indicate an environmental degradation of deltamethrin, probably due to the presence of microorganisms in soil capable of degrade deltamethrin.

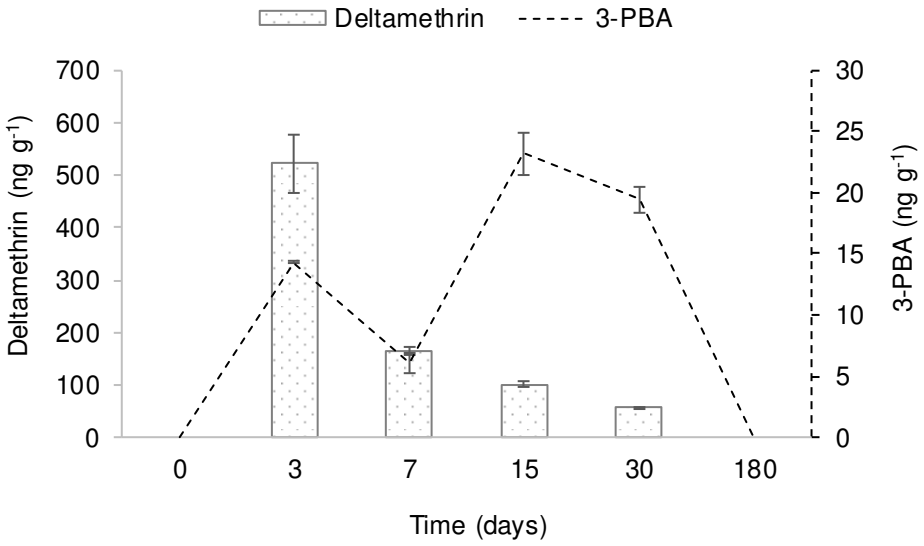

Fig. 2. Deltamethrin and 3-PBA concentration evolution in soil after initial Decis® EC 25 application.

3.3 Microbial community characterization

A total of 510,891 sequences of 16S rRNA gene were generated from the 12 samples processed by the NGS analysis pipeline at the SILVA rRNA gene database project. Of these, 56,898 reads (11.14% of the total dataset) were rejected at the initial quality filtering step. In all, 8754 reads with percentage similarity to the closest relative below 93 in BLAST analysis were classified as 'No relative' reads (without any close relatives) and the remaining 445,239 (87.15% of the total dataset) were classified (Table S1).

All sequence libraries were far from saturation as shown by the rarefaction curves (Fig. S1 supplementary data) indicating that higher number of sequences is apparently required to cover the whole community diversity.

Soil microbial community was assessed along with the determination of the different analytes. Since similar pattern were observed within each treatment, the average of two replicates was considered. The prokaryotic community was firstly described at higher taxonomic level (considering the most abundant phyla, with relative abundance >1%). The soil samples over time showed little differences in terms of dominant groups of bacteria but significant differences in the group's quantities (Fig. 3). A trend could be found between samples collected at day zero and day 30th (G0 and G30). G0 soil was dominated by the phylum Proteobacteria (35%), Bacteroidetes (12%), Actinobacteria (10%) Acidobacteria (10%), Planctomycetes (7%) and Chloroflexi (5%). For G30 sample a significant increase was observed for Actinobacteria (27%), a small increase for Chloroflexi (7%), a decrease for Proteobacteria (25%), Bacteroidetes (7%), and Acidobacteria (6%) while Planctomycetes (7%) remained with the same percentage. After 180 days of deltamethrin application (G180) in terms of general taxa the microbial community composition seems to recover to similar percentages as the ones observed in the initial soil. Zang et al. (2006) found that for methyl parathion insecticide contaminated soils, the dominant phylotypes (bacillus genus, and α-proteobacteria) were replaced by a member of the *Flexibactera cytophaga*- bacteroides and two members of the γ -proteobacteria.

A more detailed composition of the most abundant taxa (> 0.5%) present in soils over time after deltamethrin application can be found in Table 3. Most of the OTUs (Operational Taxonomic Units) assigned are below 0.5%, but we will only consider the most abundant ones. There are few most abundant OTUs and a long tail of low-abundance species in these soil samples.

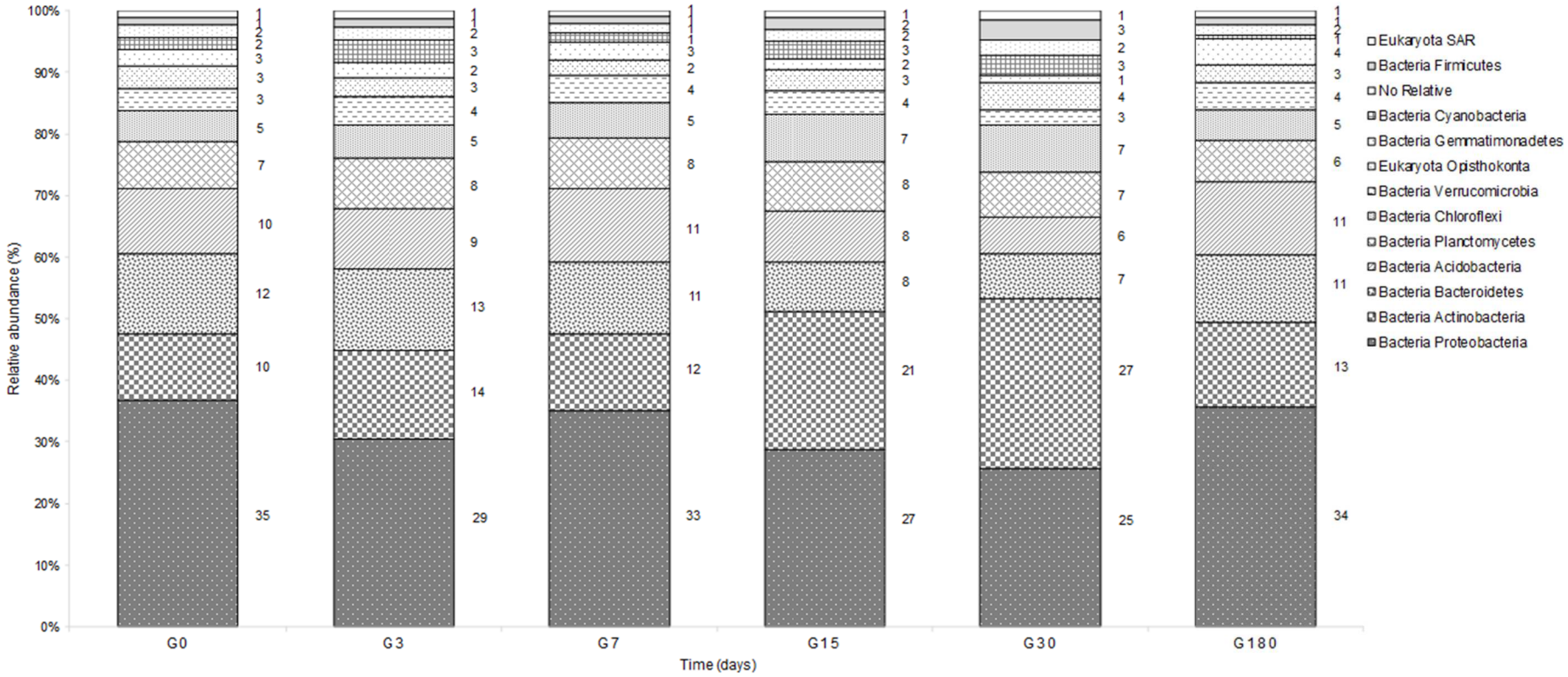

Fig. 3. Percentage of the most abundant phyla (relative abundance >1%) present in soils over time after deltamethrin application

Recent advances in next-generation sequencing technologies revealed that microbial communities are characterized by a few dominant taxa in high abundance and a long tail of rare taxa in low abundance (Sogin et al., 2006; Pedrós-Alió, 2007; Galand et al., 2009). The correlation between deltamethrin and 3-PBA concentrations along time with the microbial community can reveal the potential of the microorganisms to biodegrade such compounds. The most striking differences in microbial diversity by comparison with the initial conditions can be observed after 30 days, were *Nocardioides* became the most abundant genus. *Nocardioides* increased abundance correlates well with deltamethrin disappearance along time. This same Actinobacteria had a significant increase in the microbial communities abundance in presence of difficult compounds to degrade, such as hexachlorobenzene (Takagi et al., 2009) and is also well known to degrade the herbicide chloro-s-trazina (Ortiz-Hernández et al., 2013). In fact, a patent with a new method that comprises *Nocardioides* sp. to degrade toxic compounds such as fungicides, herbicides that also included a synthetic pyrethroid insecticide was register in 2008 (Coppin et al., 2008). Actinobacteria in terrestrial ecosystems play relevant ecological roles including recycling of substances has it was demonstrated their ability to bioremediate organic and inorganic contaminants and to produce bioactive molecules (Alvarez et al., 2017). Other Actinobacteria, are promising candidates for biodegradation such as members of the genus *Rhodococcus* that can be particularly helpful in the degradation of less soluble pesticides due to their hydrophobic nature (Kolekar et al., 2014). *Rhodococcus* sp. was able to efficient degrade atrazine (Fazlurrahman et al., 2009) and the insecticide endosulfan (Verma et al., 2006; Verma et al., 2011).

Among Proteobacteria, two of the most represented genera were *Massilia* and *Sphingomonas* that present an initial decrease followed by a peak of abundance 30 days after the exposition. A novel species in the genus *Massilia* was isolated from soil and was recognized as an herbicide-degrading bacterium (Lee et al., 2017), while *Sphingomonas* has been recognized to completely degraded the metabolite 3-PBA (Tang et al. (2013).

The phylum Firmicutes also presented an increase 30 days after the exposition, particularly notorious for the genus Bacillus. Firmicutes are frequently used in anaerobic sludge blanket reactors where they are well known as propionate-degrading bacteria (Narihiro et al., 2012). *Bacillus* has been recognized before by its ability to completely degraded the metabolite 3-PBA (Chen at al. (2012a). In addition, *Bacillus* was described by Huang et al. (2010) and Liu et al. (2015) to be able to catabolize the pyrethroid pesticide fenpropathrin and recognized by its potential in bioremediation on soils contaminated of pyrethroids.

Table 3

Percentage of the most abundant taxa (abundance >0.5 %) present in initial soils and after 3, 7, 15, 30 and 180 days of deltamethrin application. Intensity of the shade of the values increases with increasing percentages of sequences of the most abundant taxa.

Phylum	Class	Order	Family	Genus	G0	G3	G7	G15	G30	G180
Acidobacteria	Subgroup 6				4.1	3.5	4.2	3.0	1.7	4.0
	Holophagae	Subgroup 7			0.9	0.7	0.9	0.5	0.3	1.0
	Thermoanaerobaculia	Thermoanaerobaculales	Thermoanaerobaculaceae	*Subgroup 10*	0.3	0.7	0.6	0.8	0.8	0.4
	Blastocatellia (Subgroup 4)	Pyrinomonadales	Pyrinomonadaceae	*RB41*	0.6	0.5	0.7	0.4	0.3	0.8
	Acidobacteriia	Acidobacteriales	uncultured		0.5	0.4	0.5	0.3	0.2	1.3
Actinobacteria	Actinobacteria	Propionibacteriales	Nocardioidaceae	*Nocardioides*	1.0	1.6	1.1	2.9	4.4	1.3
				Marmoricola	0.1	0.5	0.4	0.7	1.2	0.3
		Micrococcales	Micrococcaceae	*Arthrobacter*	0.5	1.0	0.6	0.9	1.1	0.5
		Corynebacteriales	Mycobacteriaceae	*Mycobacterium*	0.4	0.5	0.4	0.9	0.9	0.5
		Frankiales	Nakamurellaceae	*Nakamurella*	0.3	0.4	0.4	0.7	0.9	0.3
	Thermoleophilia	Gaiellales	uncultured		0.9	1.2	1.1	1.9	1.9	1.8
			Gaiellaceae	*Gaiella*	0.6	0.7	0.7	1.2	1.1	0.6
		Solirubrobacterales	67-14		0.6	0.8	0.6	1.8	2.0	1.0
	Acidimicrobiia	IMCC26256			0.5	0.6	0.7	0.8	0.7	0.6
Bacteroidetes	Bacteroidia	Chitinophagales	Chitinophagaceae	*uncultured*	2.1	2.2	2.0	1.3	1.5	1.7
				Ferruginibacter	1.7	1.8	1.2	0.9	0.9	0.9
				Terrimonas	1.1	0.9	1.0	0.8	0.4	0.7
		Cytophagales	Microscillaceae	*uncultured*	0.6	0.8	0.8	0.5	0.3	0.9
		Flavobacteriales	Flavobacteriaceae	*Flavobacterium*	1.7	1.2	1.2	0.4	0.4	2.0
Chloroflexi	KD4-96				0.7	0.8	0.8	1.5	1.6	0.8
	Chloroflexia	Chloroflexales	Roseiflexaceae	*uncultured*	0.5	0.6	0.5	0.8	1.0	0.4
	Ktedonobacteria	C0119			0.4	0.5	0.4	0.7	0.9	0.3
	Anaerolineae	SBR1031	A4b		0.6	0.5	0.6	0.8	0.5	0.3
Cyanobacteria	Oxyphotobacteria	Chloroplast			0.7	1.9	0.8	1.5	2.1	0.3
Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	0.4	0.6	0.4	0.7	1.5	0.3
Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	*uncultured*	1.5	1.3	1.7	1.0	0.5	2.9
				Gemmatimonas	0.7	0.7	0.7	0.5	0.3	0.8
Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	*Nitrospira*	0.4	0.4	0.8	0.5	0.3	0.9
Opisthokonta	Nucletmycea	Fungi	Mucoromycota	*Mortierellomycotina*	0.7	0.8	0.6	0.6	0.7	0.6

Table 3

(continued)

Planctomycetes	Phycisphaerae	Tepidisphaerales	WD2101 soil group		1.5	1.7	1.4	1.1	1.0	1.6
	Planctomycetacia	Gemmatales	Gemmataceae	*uncultured*	0.8	1.2	1.3	1.6	1.6	0.8
		Pirellulales	Pirellulaceae	*Pir4 lineage*	0.9	0.8	0.7	0.6	0.5	0.6
				Pirellula	0.7	0.8	0.6	0.8	0.7	0.6
				uncultured	0.6	0.6	0.7	0.6	0.4	0.5
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Sphingomonas*	3.4	1.9	2.2	2.1	3.1	1.8
		Rhizobiales	Xanthobacteraceae	*uncultured*	1.0	0.8	1.0	1.0	0.6	1.0
				Bradyrhizobium	0.8	0.7	0.6	0.6	0.4	0.7
	Deltaproteobacteria	Myxococcales	Haliangiaceae	*Haliangium*	0.6	0.6	0.6	0.5	0.3	0.8
	Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae	*Massilia*	1.6	1.3	0.8	0.8	2.1	0.9
			Nitrosomonadaceae	*Ellin6067*	1.5	1.0	1.2	0.8	0.3	1.4
			Nitrosomonadaceae	*MND1*	0.7	0.7	1.2	0.8	0.3	1.9
			SC-I-84		1.1	0.8	1.1	0.6	0.4	1.0
Verrucomicrobia	Verrucomicrobiae	Pedosphaerales	Pedosphaeraceae		1.0	2.0	1.9	1.8	1.2	1.4
		Chthoniobacterales	Chthoniobacteraceae	*Chthoniobacter*	0.8	0.6	0.5	0.5	0.3	0.8
				Candidatus Udaeobacter	0.6	0.6	0.6	0.4	0.3	0.9
No relative					2.0	1.9	1.4	1.9	2.4	1.7

A hierarchical clustering (Fig. 4) was performed to analyse community structure changes over time. In general, three main clusters were observed, one formed by the initial samples (G0, G3, G7 and G15), another formed by the samples after 30 days of application (G30) and a third cluster formed by the microbial consortia at the end of the monitoring (G180). In general, the replicate samples were grouped together, showing a good replication procedure.

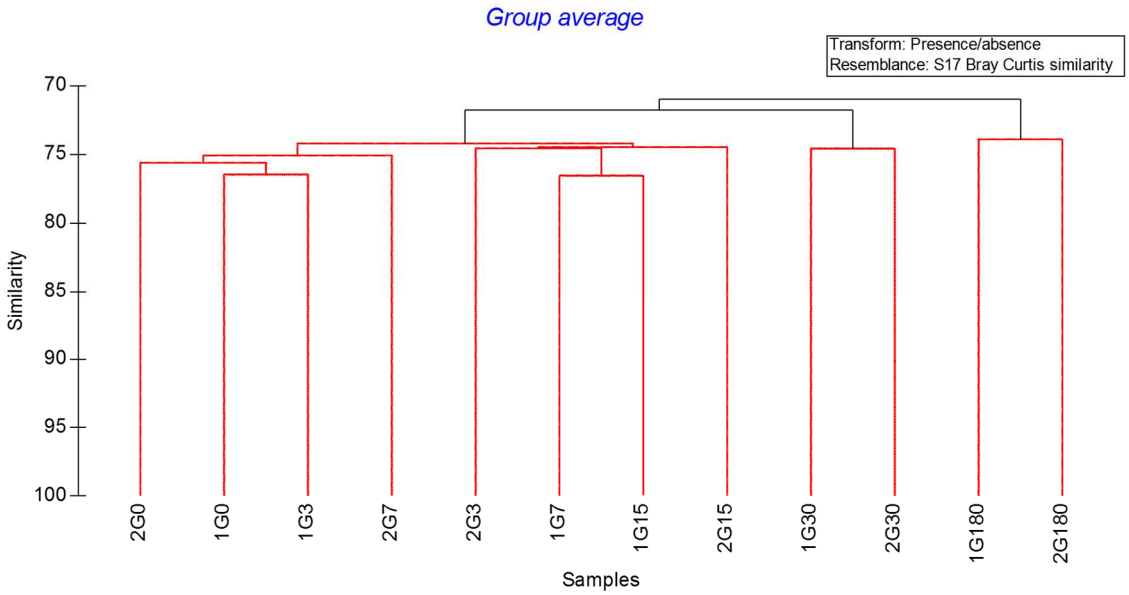

Fig. 4. Hierarchical clustering based on Bray-Curtis similarities of NGS fingerprints. G0 represents the initial soil before deltamethrin application, G3, G7, G15, G30 and G180 represent the soil after 3, 7, 15, 30 and 180 days of deltamethrin application, respectively. Numbers before the “G” letter represent replicate samples.

Overall, deltamethrin application caused a shift in the microbial community structure of the soil which was more notorious 30 days after the pesticide application. Although in terms of general taxa (phyla) the relative abundance seems similar between the beginning and the end of the experiment, hierarchical clustering revealed that after 180 days the microbial community is still different from the initial. Deltamethrin cause an environmental impact much further than the 7 days security interval described in the insecticide label.

4. Conclusions

The impact of deltamethrin on soil, after a commercial formulation application on Portuguese cabbage, was sensitively and effectively assessed by analysing the deltamethrin levels over time in cabbage and soil. The presence of deltamethrin metabolite, 3-PBA, was also monitoring in soil. Simultaneously, the soil microbial diversity over time was assessed. The monitoring of deltamethrin in cabbage and soil have a similar decay in the first three days and disappearing almost completely after 180 days. The degradation half-life time ($T_{1/2}$) in soil was equal to 8.8 days and degradation rate was 0.079 day^{-1}. Results clearly showed that, deltamethrin application caused an increase of the 3-PBA metabolite with a maximum concentration value of 23.2 ng g^{-1} after 15 days. Deltamethrin seems to cause a shift in the microbial community structure of soil which was noticed after 30 days. At this time, bacteria recognized to degrade pyrethroid and its metabolite such as *Nocardioides sp*. and *Sphingomonas sp*. had a significant increase. After 180 days of the application it was seen that the microbial community is still different from the initial one. So, deltamethrin, may cause an environmental impact much longer than the security interval. Although, more research is needed to better understand processes by which microorganisms potentiate natural degradation, the present study also shows that natural degradation can be an important technique for pesticides soil decontamination.

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Appendix A. Supplementary data

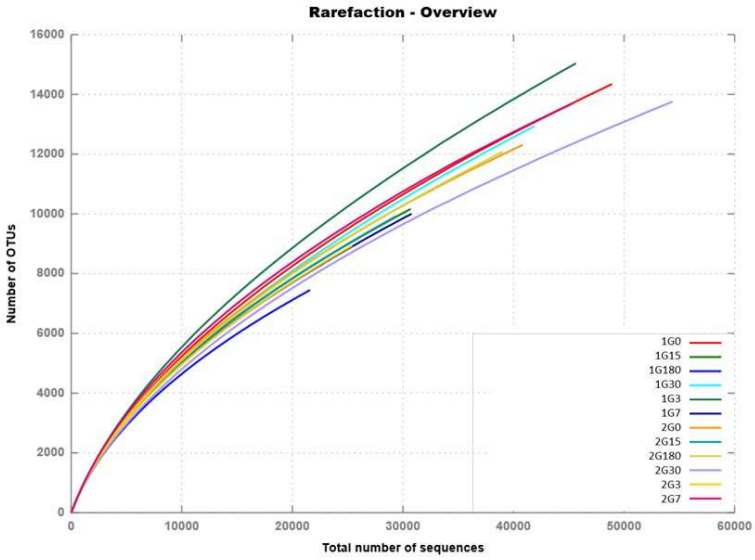

Fig. S1. Rarefaction curves obtained using OTUs at 98% similarity

Table S1

Summary statistics of 16S rRNA gene amplicon sequencing.

Sample ID	Number of sequences	# OTUs[a] (%)	% Classified	% No Relative	% Rejected
1G0	53134	14344 (27.00)	90.06	2.00	7.94
2G0	46705	12308 (26.35)	85.90	1.57	12.53
1G3	49609	15037 (30.31)	89.87	2.14	7.99
2G3	28602	8942 (31.26)	87.54	1.36	11.10
1G7	35252	10003 (28.38)	86.08	1.27	12.65
2G7	52138	13695 (26.27)	85.72	1.20	13.08
1G15	33883	10165 (30.00)	88.76	1.86	9.38
2G15	33107	9926 (29.98)	87.19	1.59	11.21
1G30	47562	12938 (27.20)	85.79	2.31	11.90
2G30	61538	13759 (22.36)	86.54	1.85	11.61
1G180	24446	7451 (30.48)	86.96	1.42	11.62
2G180	44915	12084 (26.90)	85.37	1.55	13.08
Total	510891	140652 (27.53)	87.15	1.71	11.14

[a]Operational taxonomic unit at 2% sequence dissimilarity

CHAPTER 4

Phytotoxicity of pyrethroid pesticides

Phytotoxicity of pyrethroid pesticides and its metabolite towards *Cucumis sativus*

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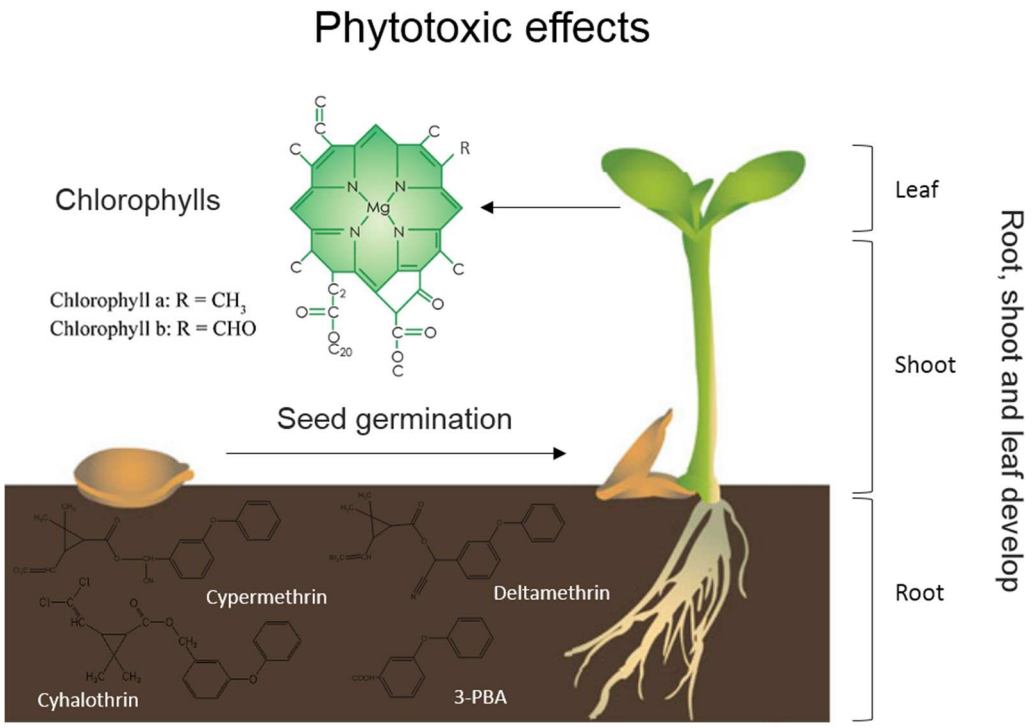

Statement of contribution

The contribution of the candidate, Idalina Bragança, in this work includes the accomplishment of all the experimental work, that is, the acute toxicity seedling growth tests, chlorophylls and carotenoids extraction and determination, statistical analysis and paper writing.

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Phytotoxicity of pyrethroid pesticides and its metabolite towards *Cucumis sativus*

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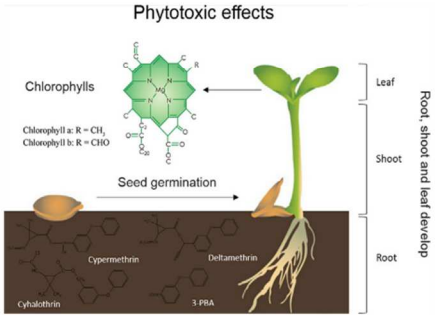
4

HIGHLIGHTS

- Potential phytotoxicity of pyrethroids and 3-PBA to soil.
- Pyrethroids and 3-PBA effect to *Cucumis sativus* seeds germination.
- Negative impact of cypermethrin and deltamethrin on seed development.
- Chlorophyll and carotenoids showed to be sensitive to some pyrethroids exposure.

GRAPHICAL ABSTRACT



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ABSTRACT

Pyrethroid pesticides residues have been frequently detected in soils and have been recognized to contribute to soil toxicity. The phytotoxic impact of pesticides was evaluated in *Cucumis sativus* (*C. sativus*) seeds. Percentage of seed germination, root elongation, shoot length and leaf length were considered as endpoints to assess the possible acute phytotoxicity of soil by the exposure to pyrethroid pesticides (cypermethrin, deltamethrin and cyhalothrin) and its metabolite phenoxybenzoic acid (3-PBA), in a concentration range between 50 and 500 μg kg^{-1}. For germination percentage, it was only observed a negative impact when seeds were exposed to the metabolite. Cypermethrin showed impact in the three studied endpoints of seed development, while deltamethrin merely affected the root length. Concerning pigments content, it can be said that chlorophylls and total carotenoids median values increased for cypermethrin and deltamethrin. This increase was more pronounced to deltamethrin in joint effect with the organic solvent dimethyl sulphoxide (DMSO). When exposed to cyhalothrin and 3-PBA, no statistically significant differences were observed for *C. sativus* seeds to all the assessed endpoints of seed development and the investigated pigments content. This research brings new data concerning the relative sensitivity of *C. sativus* seeds to pyrethroids pesticides commonly found in agricultural facilities, as well as critical understanding and development of using *C. sativus* for phytotoxicity assessments efforts for pesticide exposures.

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1. Introduction

Pyrethroids pesticides are widely used as insecticides in agriculture, veterinary and domestic applications to control insect pests (Albaseer et al., 2011; Bronshtein et al., 2012). Synthetic pyrethroids were developed to preserve the insecticidal activity of pyrethrins and to enhance physical and chemical properties, as the stability to light (Gosselin et al., 1984). Pyrethroids differ from many other pesticides in that they have three typical characteristics: extreme hydrophobicity, rich stereochemistry (contain one to three chiral centers) and broad-spectrum high-level insecticidal activity. These pyrethroids represent a significant improvement when compared to other insecticide classes due to their very low non-target mammals and birds toxicity and better selectivity to target species (Luo and Zhang, 2011). The pyrethroids are the most common active ingredients in commercially available insect sprays and are the dominant pesticides for malaria control (Raghavendra et al., 2011; Ranson et al., 2011). Depending on the type of soil and the initial concentration the half-lives of pyrethroids in soil have been estimated to be between 17.1 and 52.1 and 8.3–105.3 days for cypermethrin and deltamethrin, respectively (Cycoń and Piotrowska-Seget, 2016).

Pyrethroids in the terrestrial environment occur mainly via spray drift but accidental spills and direct application to soil can also arise (Palmquist et al., 2012). Pyrethroids dynamic in soils is dependent on soil physical-chemical characteristics. Pyrethroids strongly bind to soil particles and organic matter due to their highly hydrophobic properties (Gu et al., 2008; Oudou and Hansen, 2002; Xu et al., 2015). These pesticides residues have been frequently detected in soils and sediments and have been recognized to contribute to sediment toxicity. Sediments in California's Central Valley were tested and in three-fourths of the toxic samples, pyrethroids exceeded concentrations expected to cause toxicity. Bifenthrin reached a maximum of 32 μg kg^{-1} in sediment much higher than the LC_{50} of 3–10 μg kg^{-1} for some invertebrates (*Hyalella azteca* or *Chironomus dilutus*). This study (Weston et al., 2013) reaffirms the contribution of pyrethroids to sediment toxicity even though sampling was not specifically focused on areas of high pyrethroids use. Pyrethroids were detected at maximum concentrations of 57.0 μg kg^{-1} before plow and 62.3 μg kg^{-1} during rice production in the soil in Mediterranean paddy fields, being resmethrin and cyfluthrin the compounds found at higher concentrations (Aznar et al., 2017). Pyrethroids presence was much higher in the sediments of creeks within a residential neighborhood near Roseville (Weston et al., 2005), where cyfluthrin, permethrin, bifenthrin, and cypermethrin were found at maximum concentrations of 169, 335, 437 and 736 μg kg^{-1}, respectively.

Pyrethroids in soil follow different pathways such as degradation, sorption-desorption, volatilization, uptake by plants and can also be transported into surface waters and groundwaters (Cycoń and Piotrowska-Seget, 2016). Microbial degradation of pyrethroids appears to be a significant breakdown route of such pesticides (Palmquist et al., 2012). The main process of environmental degradation of pyrethroids consists in the hydrolysis of carboxylester linkage that results most frequently in the production of cyclopropane acid and an alcohol moiety (3-phenoxybenzyl alcohol). The 3-phenoxybenzyl alcohol is rapidly converted to 3-phenoxybenzoic acid (3-PBA). 3-PBA frequently accumulates in the soil as well as the others metabolites referred above (Chen et al., 2011). Tyler et al. (2000) found that cyclopropane, permethrin acid, 3-phenoxybenzyl alcohol and 3-phenoxybenzoic acid, metabolites of environmental permethrin pyrethroid degradation, are of greater concern than their parent compound owing to their ability to interact with steroids hormone receptors. These metabolites in the environment can modulate and/or disrupt the endocrine systems of animals.

Pyrethroids raise environmental concerns due to their increasing and intensive use and potential effects on aquatic ecosystems as they are highly toxic to fish and invertebrates (Antwi and Reddy, 2015; Haya, 1989; Zhang et al., 2011). There are many studies documenting toxicological effects of pesticides on plants but studies tended to focus on herbicide effects in crop species (Gomes et al., 2017; Wagner and Nelson, 2014).

The increasing and widening use of pyrethroid insecticides cannot only cause residues in the soil but even lead to detrimental effects on plants and other non-target organisms, which necessitates a thorough understanding of their phytotoxicity. Research on the effects of pyrethroids on seed germination are scarce (Hanley and Whiting, 2005; Moore and Locke, 2012). Subsequently, a phytotoxic impact of these pesticides on soil should be assessed. Plant damage due to the application of pesticides is known as phytotoxicity. Pesticide phytotoxicity appears in several ways on plants, causing the studied compound an impact on plant characteristics. Plants are at their most sensitiveness to chemical's application during the early stages of life (Hewitt and Rennie, 1986). Inhibition of germination or on the root, shoot, and leaf development are the main areas of interest in studies on phytotoxicity (Kapanen and Itävaara, 2001). Seed germination and development can be affected by pyrethroid pesticides application. Hanley and Whiting (2005) reported that deltamethrin decreased the seedling growth of *Capsella bursa-pastoris* L. and *Poa annua* L. With increasing concentrations of cypermethrin and fenvalerate the germination rates of *Pennisetum pedicellatum* Trin showed a tendency to decrease (Dubey and Fulekar, 2011). Germination percentages of primary roots of *Glycine* max L. decreased with increasing cypermethrin concentration (Aksoy and Deveci, 2012). Visible phytotoxic effects can also occur, as chlorosis, necrosis, wilting and leaf and stem deformations (Wang and Williams, 1988). Cucumber and lettuce are the biological test species recommended by the U.S. Environmental Protection Agency for toxicity testing and environmental assessment (USEPA, 1996). The terrestrial plants assays for testing chemicals are considered valid when the following performance criteria are met for water controls: the seedling emergence must be at least 70%; the seedlings do not exhibit visible phytotoxic effects (e.g. chlorosis, necrosis, wilting, leaf and stem deformations); the plants exhibited only normal variation in growth and morphology for that species, and the mean survival of emerged control seedlings is at least 90% for the duration of the study (OECD, 2006).

The toxicity and the ecological risks in terrestrial environments of the mixtures of organic pollutants can be ascertained by the inhibition of photosynthesis efficiency (Gonzalez-Naranjo et al., 2015). Photosynthesis sustains nearly all life on Earth since it is largely responsible for providing oxygen present in Earth's atmosphere (Johnson and Portland Press, 2016). The measure of Chlorophyll-*a* (Ch *a*), Chlorophyll-*b* (Ch *b*) and total carotenoids (C x + c) are powerful tools to determine photosynthetic activity and so to evaluate the stress impact in plants (Zarco-Tejada et al., 2009). External factors can undesirable change and influence plants, causing different physiological responses (Ibanez et al., 2010). The Chlorophyll *a*/*b* ratio ($Ch_{a/b}$) is a conceivable indicator of nitrogen partitioning within a leaf since it can be positively correlated with the ratio of photosystem II cores to light harvesting chlorophyll-protein complex (Hikosaka and Terashima, 1995). Carotenoids and anthocyanins have also to be considered since they are the main pigments known to be involved in protecting plant organs from stress. Also, carotenoids additionally function as non-enzymatic antioxidants (Strzalka et al., 2003).

The aim of this study was to evaluate the phytotoxic effects of three synthetic pyrethroids (cypermethrin, deltamethrin, and cyhalothrin) and of one of their metabolites (3-PBA) in *Cucumis sativus* (cucumber) seeds. Given the sensitivity of seeds to chemicals, the effects of different concentrations (50, 125, 200, 350 and 500 μg kg^{-1} soil dry weight) of these compounds on seed germination, subsequent early growth of seedlings and pigments content of leafs were the main parameters followed. To the best of the authors' knowledge, this is the first study on the pyrethroid pesticides (cypermethrin, deltamethrin, and cyhalothrin) and its metabolite (3-PBA) phytotoxicity to *C. sativus* germination, the seedling grown and photosynthesis.

2. Materials and methods

2.1. Study pesticides

For this study, three pyrethroid pesticides and its major metabolite were used: α-cypermethrin, ʎ-cyhalothrin, deltamethrin, and 3-PBA. Pesticides and metabolite standards (purity ≥95.4%) were obtained from Chemservice (West Chester, PA, USA) and Sigma-Aldrich Co. (Steinheim, Germany), respectively. The dimethyl sulphoxide (DMSO) (p.a., dried) was also obtained from Sigma-Aldrich Co. A range of pesticides concentrations was chosen for this study in anticipation of generating comparative-response data. Stock solutions of pyrethroids and 3-PBA were prepared in DMSO due to their insolubility in water (concentrations ranging between 600 and 700 ppm) and stored in the dark at 4 °C. This polar aprotic solvent is less toxic than other members of its class and is commonly used in biological tests as a vehicle for non-water-soluble chemicals (Yu et al., 2017). DMSO was utilized in a small percentage (0.4%) and a control setting for DMSO was used to observe if the effects in the treated seeds were due to the chemical and not to the solvent/vehicle. The five working solutions used to contaminate the soils were prepared daily by dispersing amounts of stock solutions in water obtaining the following concentration: 0.250, 0.625, 1.00, 1.75 and 2.50 mg L^{-1} for each pyrethroid and 3-PBA. The DMSO amount placed in each working solution was the same, 0.4%. DMSO was used in all assays (including chlorophyll extraction) because many authors reported the merits of this solvent with high efficiency also in pigments extractions (Sumanta et al., 2014) even when concentrations were too low (Porra, 2002; Wright et al., 1997).

2.2. Test organism and artificial soil matrix

Cucumber seeds, *Cucumis sativus* var. marketmore, were acquired from Casa Hortícola (Porto, Portugal). The seeds were kept in their original paper packages and stored in the dark in a zip-lock sealed bag at 4 °C.

A certified artificial sandy soil from MIBAL - Minas de Barqueiros S.A. (Apúlia, Portugal) was used to perform all the toxicity tests. Artificial sandy soils are used for testing chemicals when a minimal variability of the natural soil is desired and to increase the comparability of the several test results (OECD, 2006). This sandy soil is predominantly siliceous with grain dimensions above 1.4 mm and composed mainly of Quartz. The soil was physical-chemical characterized according to OECD (2006) guidelines. The procedures for physical-chemical characterization (total organic carbon (TOC), water holding capacity (WHC), pH and conductivity values, organic matter (OM) content) of the sandy soil used was previously reported by Braganca et al. (2012) and Margesin and Schinner (2005).

2.3. Acute toxicity seedling growth test

Acute ecotoxicity tests were prepared according to the recommended condition and procedures for conducting reference toxicity tests on soil using terrestrial plants (EPS 1/RM/45 report, Canada) and to OCDE (2006) guidelines for the testing of chemicals.

For acute ecotoxicity tests in the soil, the concentrations of pesticides and 3-PBA used were 50, 125, 200, 350 and 500 μg kg^{-1} soil dry weight. Germination assays were carried out by placing ten replicates of five individual *C. sativus* seeds per replicate, for a total of 50 seeds per treatment. Tests were conducted using 140 × 20 mm disposable plastic Petri dishes with 100 g of pre-dried sandy soil. The soil was moistened with either 20 mL (~70% of water-holding capacity) of deionised water (Water controls), or deionised water with 0.4% of DMSO (DMSO controls) or with treatment solutions using a Pasteur pipette (dropwise in soil surface simulating a watering system) being Petri dishes sealed with parafilm. The Petri dishes were arranged in a completely random order, placed in a growth chamber (Binder, KBWF 240, USA), equipped with daylight fluorescent lamps (10,000 lx per light cassette), during a total of 168 h. The temperature used for both the first 48 h carried out in darkness, and the following photoperiods of 16 h of light (simulating day) was 24 ± 2 °C, the lasts alternated with 8 h of darkness at 15 ± 2 °C (simulating night). After seven days of exposure, the germination parameters (root, shoot, and leaf length) were measured with a certified electronic digital caliper (Paget Trading Ltd., Woodrow London SE18 5DH, UK).

2.4. Chlorophylls and carotenoids

All the fresh leafs sampled per Petri dish (equivalent to one replicate) were removed with a scissor, weighted, and extracted with solvent added in the proportion of 2 mL of DMSO per 25 mg of fresh leaf. The sample was homogenized for 15 min in the ultrasonic bath (Sonorex Digital 10P, Bandelin) (Giri et al., 2013). The chlorophyll extract was later analysed using 96-well plates, in a Synergy HT W/TRF Multimode Microplate Reader (BioTek Instruments, Winooski, VT, USA) at 480,0 649.1 and 665.1 nm against DMSO blank. The quantification of Chlorophyll-*a* (Ch_a), Chlorophyll-*b* (Ch_b) and carotenoids (C_{x+c}) was performed by using the Eqs. (1), (2) and (3), respectively (Wellburn, 1994):

$$Ch_a = 12.47A_{665.1} - 3.62A_{649.1} \quad (1)$$

$$Ch_b = 25.06A_{649.1} - 6.5A_{665.1} \quad (2)$$

$$C_{x+c} = (1000A_{480} - 1.29Ch_a - 53.78Ch_b)/220 \quad (3)$$

2.5. Statistical analysis

Data from acute ecotoxicity tests were tested for normality (Shapiro-Wilk). The non-parametric test Kruskal–Wallis was applied due to the non-normal distribution of the data with a significance threshold of $p < 0.05$. After that, a post-hoc pairwise comparison using the Dunn-Bonferroni approach was applied. Data were expressed as median and range. All statistical analyses were performed with SPSS software, version 20.0 (SPSS Inc., Chicago, Illinois).

3. Results and discussion

3.1. Germination

The knowledge of the properties of the soil exploited in agricultural activities is important considering soil management and plant growth. For the specific soil used in these assays the parameter values determined were: conductivity (8.47 ± 0.01 mS m^{-1}), pH (4.9 ± 0.3), WHC (33.5 ± 0.06%), TOC (0%) and OM (0.07 ± 0.01%) in the average range for this type of sandy soil.

Concerning the toxicity tests performed, *C. sativus* seeds germination percentages were not affected when exposed to increased pyrethroids pesticides concentrations between 50 and 500 μg kg^{-1} (Fig. 1) and also not significantly different for the several pesticides tested. The median percentages of germination for this study were 100, 80 and 100 for cypermethrin, deltamethrin, and cyhalothrin, respectively, being no significant differences in germination observed between controls and exposed pyrethroids seeds. The range of germination values (%) had a lower bound for almost all the concentration tested, however, no trend was found between germination and increasing levels of pesticides. Similar findings were reported by Moore and Locke (2012) to *Typha latifolia* when exposed to three pesticides, one of them a pyrethroid (permethrin). Hanley and Whiting (2005) determine the toxicological effects of deltamethrin and dimethoate to six species of weeds. They found that the applied insecticides had no significant effect on

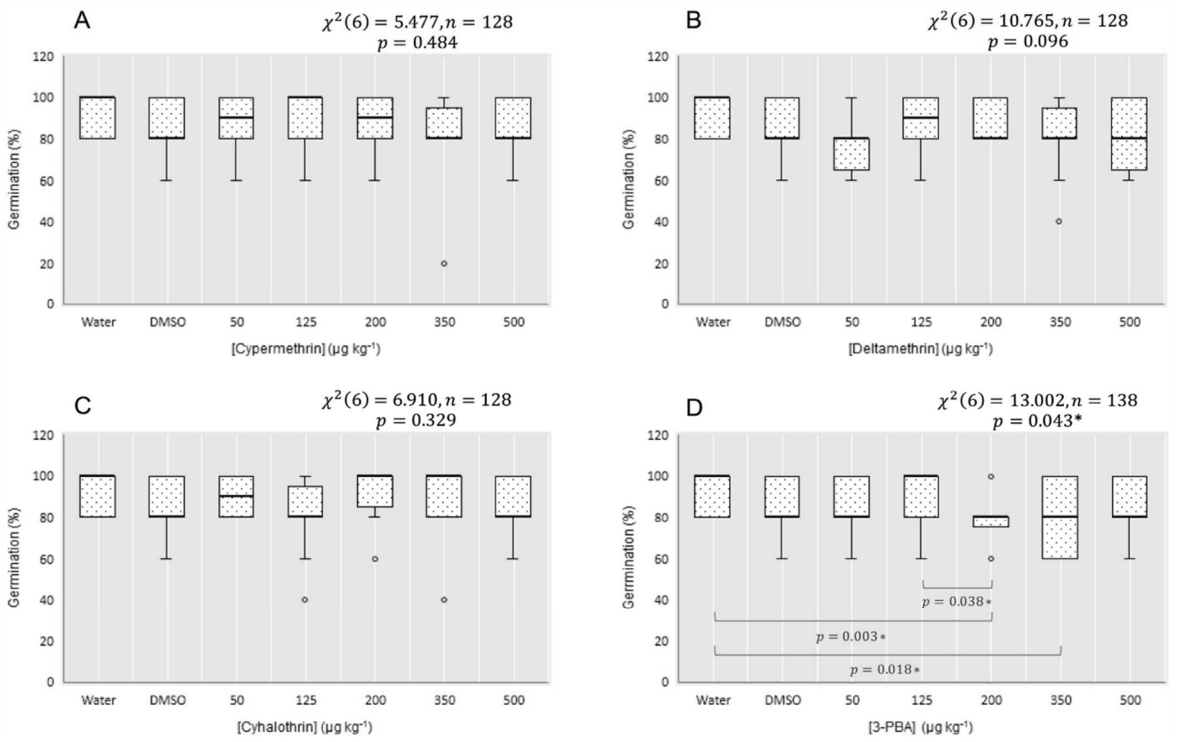

Fig. 1. Germination percentages of *C. sativus* seeds exposed to different pyrethroids pesticides (A- Cypermethrin, B- Deltamethrin and C- Cyhalothrin) and 3-PBA (D) concentrations (50, 125, 200, 350 and 500 µg kg^{-1}). * Statistically significant differences for the given medians (▬) (p value < 0.05). Two controls (Water- deionised water control and DMSO- deionised water with 0.4% of DMSO solvent control) were used to evaluate differences in seed development.

seed germination but for *Capsella bursa-pastoris* and *Poa annua* seedling growth was decreased by exposure to deltamethrin.

For 3-PBA it was possible to observe significant differences in seed germination distribution across categories, considering the concentrations used ($p = 0.043$). Differences were found between the Water control and 3-PBA concentrations of 200 µg kg^{-1} ($p = 0.003$) and 350 µg kg^{-1} ($p = 0.018$) while no differences were found for the same concentrations and DMSO control. It's likely that the combined effect of DMSO and 3-PBA concentration was most toxic to seed germination that the single effect of this compound. Since no statistically significant differences were found between the Water and DMSO controls no specific impact can be attributed to DMSO at the concentration used. Kumar et al. (2013) also used DMSO to dissolve permethrin. The toxicity of the pesticide was evaluated after its dispersion in water or by means of a nanoformulated permethrin, being applied to various non-target organisms including the *Cucumis sativus*, where DMSO also showed no significant effect on both root elongation and germination rate (%).

3.2. Root, shoot and leaf development

Commercially used pesticides, such as dimethoate and deltamethrin, are known to exert phytotoxic effects on both plant germination and growth, impacting enzymatic pathways involved in such processes (Hanley and Whiting, 2005). Kapanen and Itävaara (2001) concluded that statistically significant changes in seedling grown tests compared to root, shoot or leaf lengths for any of the tests exposed to composted materials indicate a phytotoxic effect with the same cause. Table 1 shows the results obtained for phytotoxic effects in *C. sativus* seeds development (root, shoot and leaf length) after the application of different pyrethroid insecticides concentrations. Although germination was relatively unaffected by insecticide as previously mentioned, seedling growth was significantly changed by insecticide application. The results suggested that *C. sativus* seed growth may be extremely sensitive to some pyrethroids application, in this case for three out of the four compounds tested, being some parameters more sensitive than others. Seed germination tests measure soil toxicity directly, while root elongation, shoot, and leaf length tests consider the indirect effects of water constituents that may be present in the environment. Many studies indicated that the root system was more sensitive to environmental toxicity than the other considered endpoints (Liu et al., 2009; Rede et al., 2016). The median values of root elongation for Water and DMSO controls were not statistically significantly different and were equal to 55.28 mm and 58.16 mm, respectively (Table 1). For the cypermethrin concentration of 125 µg kg^{-1} the maximum median root length (64.85 mm) was obtained, showing an increase of 17% and 12% when compared to the water and DMSO controls, respectively. For the same concentration deltamethrin exhibited also the highest median difference when compared to the controls (16% and 10% for water and DMSO controls, respectively). For the metabolite 3-PBA, the differences were not statistically significant considering a significance threshold of 0.05 ($p = 0.069$) but interestingly the maximum median of root length for 3-PBA was also found for the same concentration with a value of 61.63 mm. Root elongation results were not conclusive in terms of pyrethroid pesticides toxicity. A clear difference between the pesticides concentration and the DMSO was not observed which suggests that only the combined effect of the organic solvent plus the pyrethroid pesticide had influence and not the individual effect of each compound. For the pesticides used in the current study, shoot and leaf development were

Table 1
Phytotoxic effects in *C. sativus* seeds development (root, shoot and leaf length) after exposure to different pyrethroids pesticides and 3-PBA concentrations (50, 125, 200, 350 and 500 μg kg^{-1}). Two controls (Water- deionised water control and DMSO- deionised water with 0.4% of DMSO solvent control) were used to evaluate differences in seed development.

Compound		Concentration (μg kg^{-1})	Root elongation (mm)	Shoot length (mm)	Leaf length (mm)
			Median (min-max)		
Cypermethrin	Water	0	55.28^{a} (6.83–89.70)	11.16^{a} (3.09–23.35)	11.52^{a} (5.51–20.08)
	DMSO	0	58.16^{ab} (13.06–90.60)	10.88^{a} (3.43–24.77)	11.19^{ac} (7.11–20.09)
		50	59.88^{ab} (23.49–89.43)	8.07^{b} (2.84–17.55)	10.94^{ac} (8.33–17.03)
		125	64.85^{b} (9.13–86.75)	7.56^{b} (3.85–19.02)	9.91^{bc} (6.56–17.80)
		200	57.77^{ab} (29.8–83.02)	8.12^{b} (2.41–18.46)	10.81^{c} (8.19–16.38)
		350	59.97^{ab} (2.39–88.57)	7.51^{b} (1.68–21.41)	10.14^{ac} (8.86–18.63)
		500	64.54^{b} (21.60–87.57)	7.49^{b} (3.91–21.71)	10.44^{ac} (7.90–18.72)
		Kruskal-Wallis	$\chi^2(6) = 13.247$	$\chi^2(6) = 45.798$	$\chi^2(6) = 18.872$
		Total n = 562	p = 0.039*	p < 0.001*	p = 0.004*
Deltamethrin	Water	0	55.28^{a} (6.83–89.70)	11.16 (3.09–23.35)	11.52 (5.51–20.08)
	DMSO	0	58.16^{ab} (13.06–90.60)	10.88 (3.43–24.77)	9.67 (7.11–20.09)
		50	63.80^{b} (35.71–99.49)	9.11 (4.29–24.89)	10.24 (7.52–17.80)
		125	64.16^{b} (4.94–99.68)	9.99 (4.23–23.42)	10.91 (6.67–20.95)
		200	57.65^{ab} (10.06–91.81)	10.21 (4.40–20.18)	11.37 (6.97–17.49)
		350	57.50^{ab} (26.62–103.05)	9.77 (3.33–25.26)	10.12 (7.30–19.78)
		500	59.19^{ab} (15.61–95.75)	9.84 (4.94–22.78)	10.47 (7.60–17.83)
		Kruskal-Wallis	$\chi^2(6) = 14.066$	$\chi^2(6) = 2.675$	$\chi^2(6) = 7.964$
		Total n = 554	p = 0.029*	p = 0.848	p = 0.241
Cyhalothrin	Water	0	55.28 (6.83–89.70)	11.16 (3.09–23.35)	11.52 (5.51–20.08)
	DMSO	0	58.16 (13.06–90.60)	10.88 (3.43–24.77)	11.19 (7.11–20.09)
		50	53.43 (13.14–103.49)	9.52 (3.40–26.41)	12.80 (6.84–20.33)
		125	52.48 (11.96–82.22)	11.11 (3.87–23.18)	12.03 (8.65–19.14)
		200	57.22 (13.77–97.41)	9.94 (4.63–21.45)	11.11 (8.60–17.60)
		350	58.05 (22.77–91.83)	12.17 (5.26–21.13)	12.99 (8.26–18.53)
		500	48.15 (9.26–96.54)	14.03 (3.22–26.22)	13.26 (8.30–19.40)
		Kruskal-Wallis	$\chi^2(6) = 7.904$	$\chi^2(6) = 1.769$	$\chi^2(6) = 6.845$
		Total n = 562	p = 0.245	p = 0.940	p = 0.335
3-PBA	Water	0	55.28 (6.83–89.70)	11.16 (3.09–23.35)	11.52 (5.51–20.08)
	DMSO	0	58.16 (13.06–90.60)	10.88 (3.43–24.77)	11.19 (7.11–20.09)
		50	56.46 (24.19–94.69)	13.08 (4.06–25.04)	11.55 (8.00–18.34)
		125	61.63 (28.60–105.25)	12.41 (3.26–25.13)	11.64 (7.09–19.90)
		200	57.42 (27.29–86.62)	10.69 (5.68–24.70)	11.42 (7.66–18.19)
		350	59.99 (18.26–102.68)	11.85 (4.87–23.03)	11.38 (6.26–19.49)
		500	52.61 (27.43–90.07)	11.60 (5.10–23.76)	11.42 (6.69–19.07)
		Kruskal-Wallis	$\chi^2(6) = 11.690$	$\chi^2(6) = 3.965$	$\chi^2(6) = 1.752$
		Total n = 597	p = 0.069	p = 0.681	p = 0.941

* Statistically significant differences for a p value < 0.05. The same letters (a–i) in a row that the given medians are not statistically different (p > 0.05).

only statistically significantly different for cypermethrin exposure. A decrease of 25–31% of shoot length was observed on cucumber seeds expose to different cypermethrin concentrations when compared to DMSO controls (p values < 0.006). On leaf development, a decrease in leaf length for the 125 μg kg^{-1} cypermethrin concentration when compared with the DMSO control (p = 0.001) was also observed.

Since the 125 μg kg^{-1} concentration was the one that exhibits more effect in the three endpoints of seed development this was the concentration chosen to compare the four pesticides toxicity. No differences in root length were found between the tested compounds for this concentration. For shoot and leaf lengths, it was notable a difference between cypermethrin and the other compounds (p values < 0.007) which support the toxic effects of cypermethrin on cucumber seed development when compared to the others tested compounds. In the cyhalothrin case, no statistically significant differences were observed for all the assessed investigated endpoints development (root elongation, shoot and leaf length) towards *C. sativus* seeds, suggesting that this insecticide was the least toxic to cucumber seed germination.

3.3. Chlorophylls and carotenoids

The experimental data for Ch_a, Ch_b and C_{x+c} are shown in Table 2. An increase on chlorophylls (Ch_a from 3.80 of water and 4.55 of DMSO to a maximum of 5.57 μg/mL) and total carotenoids median values (from 0.89 of water and 1.00 of DMSO to a maximum of 1.22 μg/mL) on the leaves were noticed for cypermethrin. Cucumis leaves treated with incremental doses of cypermethrin dissolved in DMSO showed a significant increase in carotenoids (p = 0.034) comparing the control with leaves treated with water (Table 2). Identical results were observed to deltamethrin with an increase on chlorophylls (Cha achieving 6.78 μg/mL) and total carotenoids median values (achieving 1.36 μg/mL) on the leaves. Chlorophylls and carotenoids content were statistically significant different (p values < 0.008) along the deltamethrin concentration distribution that seems to be due to the joint effect of organic solvent (DMSO) and the pyrethroid pesticide. In all the cases, it was observed statistically significant differences between the water and the DMSO controls. The results suggest that cypermethrin and deltamethrin application triggers mechanisms that could contribute to different protective strategies for *C. sativus* as it was previously described for *A. hortensis* and *A. rosea* when exposed to metals (Kachout et al., 2015). A reflection of this process was observed for carotenoids that may undergo a high turnover under photo-oxidative stress due to chemical quenching of singlet oxygen (Edge and Truscott, 1999).

Deltamethrin can have some effect on the system N availability, probably involving Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), PSII and/or LHCII. The $Ch_{a/b}$ ratio was 5–7% higher for different deltamethrin pesticide concentrations when compared to the DMSO control being this increase significantly different (p < 0.018). The $Ch_{a/b}$ ratio is predicted to respond to light and N availability and $Ch_{a/b}$ ratios should increase with decreasing N availability (Hikosaka and Terashima, 1995). When N supply becomes limiting under high light intensity, the proportional allocation of N to photosystem II (PSII) should increase at the cost of decreased N allocation to Rubisco. In contrast, N allocation to light harvesting chlorophyll-protein complex (LHCII) is

Table 2
Chlorophyll and carotenoids content and $Ch_{a/b}$ ratios for *C. sativus* leaves after 7 days exposed to pyrethroids and 3-PBA. Two controls (Water- deionised water control and DMSO-deionised water with 0.4% of DMSO solvent control) were used to evaluate differences in seed development.

Compound		Concentration (μg kg^{-1})	Ch_a (μg/mL)	Ch_b (μg/mL)	$Ch_{a/b}$	C_{x+c} (μg/mL)
			Median (min-max)			
Cypermethrin	Water	0	3.80 (0.88–10.20)	1.16 (0.33–2.89)	3.36 (2.68–3.75)	0.89^{a} (0.40–1.95)
	DMSO	0	4.55 (1.63–12.08)	1.36 (0.52–3.36)	3.38 (2.83–4.34)	1.00^{b} (0.53–2.15)
		50	4.80 (1.48–11.49)	1.45 (0.50–3.66)	3.25 (2.98–3.57)	1.13^{ab} (0.41–1.83)
		125	5.01 (1.86–6.88)	1.50 (0.60–1.95)	3.38 (2.96–3.63)	1.10^{ab} (0.63–1.44)
		200	5.64 (1.74–11.26)	1.57 (0.67–3.41)	3.38 (2.59–3.72)	1.20^{b} (0.64–2.08)
		350	5.67 (2.50–7.19)	1.66 (0.75–2.19)	3.42 (3.14–3.61)	1.22^{b} (0.54–1.49)
		500	5.57 (3.29–9.94)	1.58 (0.97–2.87)	3.41 (3.01–3.68)	1.22^{b} (0.75–1.91)
		Kruskal-Wallis	$\chi^2(6) = 11.322$	$\chi^2(6) = 11.697$	$\chi^2(6) = 3.725$	$\chi^2(6) = 13.635$
		Total n = 130	p = 0.079	p = 0.069	p = 0.710	p = 0.034*
Deltamethrin	Water	0	3.80^{a} (0.88–10.20)	1.16^{a} (0.33–2.89)	3.36^{a} (2.68–3.75)	0.89^{a} (0.40–1.95)
	DMSO	0	4.55^{b} (1.63–12.08)	1.36^{b} (0.52–3.36)	3.38^{a} (2.83–4.34)	1.00^{b} (0.53–2.15)
		50	6.90^{b} (2.19–12.89)	1.93^{b} (0.65–3.57)	3.56^{b} (3.24–3.73)	1.37^{b} (0.60–2.38)
		125	6.21^{b} (4.17–9.86)	1.76^{b} (1.14–2.76)	3.61^{b} (3.30–3.79)	1.26^{b} (0.94–1.84)
		200	7.40^{b} (3.12–9.62)	2.06^{b} (1.00–2.77)	3.58^{ab} (3.11–3.92)	1.41^{b} (0.78–1.79)
		350	6.10^{ab} (2.59–9.67)	1.67^{ab} (0.66–2.72)	3.54^{b} (3.26–3.91)	1.27^{ab} (0.59–1.83)
		500	6.78^{b} (2.64–10.49)	1.91^{b} (0.72–2.99)	3.57^{b} (3.32–3.77)	1.36^{b} (0.66–1.94)
		Kruskal-Wallis	$\chi^2(6) = 19.781$	$\chi^2(6) = 17.451$	$\chi^2(6) = 26.863$	$\chi^2(6) = 18.826$
		Total n = 130	p = 0.003*	p = 0.008*	p < 0.001*	p = 0.004*
Cyhalothrin	Water	0	3.80 (0.88–10.20)	1.16 (0.33–2.89)	3.36 (2.68–3.75)	0.89 (0.40–1.95)
	DMSO	0	4.55 (1.63–12.08)	1.36 (0.52–3.36)	3.38 (2.83–4.34)	1.00 (0.53–2.15)
		50	4.14 (1.61–6.26)	1.26 (0.49–1.81)	3.40 (3.18–3.78)	0.97 (0.44–1.28)
		125	4.77 (1.84–7.85)	1.42 (0.57–2.33)	3.37 (3.19–3.60)	0.99 (0.45–1.57)
		200	4.30 (3.24–10.86)	1.26 (1.10–3.01)	3.23 (2.83–3.61)	0.94 (0.78–2.01)
		350	4.57 (2.61–8.79)	1.29 (0.77–2.58)	3.38 (3.27–3.59)	1.00 (0.58–1.66)
		500	4.42 (2.91–8.68)	1.36 (0.89–2.48)	3.44 (3.20–3.76)	0.96 (0.67–1.67)
		Kruskal-Wallis	$\chi^2(6) = 7.547$	$\chi^2(6) = 8.197$	$\chi^2(6) = 2.986$	$\chi^2(6) = 6.544$
		Total n = 130	p = 0.274	p = 0.224	p = 0.811	p = 0.365
3-PBA	Water	0	3.80 (0.88–10.20)	1.16 (0.33–2.89)	3.36 (2.68–3.75)	0.89 (0.40–1.95)
	DMSO	0	4.55 (1.63–12.08)	1.36 (0.52–3.36)	3.38 (2.83–4.34)	1.00 (0.53–2.15)
		50	3.79 (1.74–6.92)	1.14 (0.37–2.02)	3.33 (3.20–4.76)	0.91 (0.53–1.44)
		125	3.60 (2.02–6.45)	1.10 (0.58–1.88)	3.39 (2.98–3.72)	0.79 (0.62–1.24)
		200	3.96 (2.75–6.05)	1.16 (0.78–1.77)	3.41 (3.28–3.62)	0.88 (0.54–1.23)
		350	4.29 (1.18–6.05)	1.24 (0.33–1.86)	3.35 (3.00–3.61)	0.92 (0.30–1.18)
		500	5.96 (1.96–8.87)	1.78 (0.55–2.53)	3.43 (3.24–3.61)	1.21 (0.57–1.72)
		Kruskal-Wallis	$\chi^2(6) = 9.109$	$\chi^2(6) = 8.978$	$\chi^2(6) = 2.759$	$\chi^2(6) = 9.556$
		Total n = 130	p = 0.168	p = 0.175	p = 0.838	p = 0.145

* Statistically significant differences for a p value < 0.05. The same letters (a-i) in a row that the given medians are not statistically different (p > 0.05).

maintained at a similar level (Kitajima and Hogan, 2003). Consequently, the ratio of PSII to LHCII (and the $Ch_{a/b}$ ratio) should increase with decreasing N availability.

For cyhalothrin and 3-PBA, the imposed stress had no effect on the pigments leaves of *C. sativus*, as no statistically significant differences were observed to all the assessed investigated pigments content.

Although the water and DSMO controls did not show significant differences the results obtained in terms of germination/growth suggest a synergistic effect of DSMO together with the pyrethroids applied. It seems likely that the organic solvent may also have an effect in chlorophylls in addition to the other tested parameters. The need for the utilization of a solvent in order to solubilize the tested compounds points to the importance of other compounds used in the formulation of the final product, other than the pesticide itself, which can have indirect effects on the Environment.

4. Conclusions

Cypermethrin, deltamethrin, and cyhalothrin are some of the most used synthetic pyrethroids in agriculture applications, namely for plants pest control. Due to their capacity to bind to soil particles and passed through plants, it's of significant importance to assess their environmental toxicity. Their metabolites, such as 3-PBA, can also cause impact on the environment. Phytotoxicity can be assessed using such parameters as germination, seedling development (root, leaf, and shoot) and chlorophyll/carotenoid contents. A direct effect of 3-PBA on germination was observed while the pyrethroids tested did not show any influence on this parameter. For the tested pyrethroids cypermethrin was the one with most impact on seedling development, namely on leaf and shoot elongation. Root elongation was more affected by deltamethrin. The impacts of the four compounds were also evaluated through the determination of chlorophyll *a*nd carotenoids contents after 7 days of exposure. Studies with deltamethrin were the ones that presented higher values for these two parameters with statistically significant differences for chlorophyll *a*, b, their ratio and for carotenoids (C_{x+c}).

The results highlight the effect that pyrethroids have on the early growth of plants. The assessed parameters, seedling germination, and development as well as chlorophyll and carotenoids contents, contributed to the knowledge of the phytotoxicity of these three different pyrethroids and their metabolite. A higher emphasis is necessary for research in commonly applied agrochemicals that affect the biology of non-target organisms, as was shown for the tested compounds.

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CHAPTER 5

Final conclusions & Future perspectives

This last chapter includes the main conclusions obtained from this thesis and presents some future perspectives.

Final conclusions & Future Perspectives

Nowadays, pyrethroid pesticides are recognized as a growing class of contaminants, due to their presence in the environment as well as their toxicity to some non-target organisms. Annually, tons of pesticides are produced and consumed worldwide. The intensive use of pyrethroid pesticides raise environmental concerns, especially regarding soil contamination.

In the present work two analytical methodologies were implemented for the determination of pyrethroids and their major metabolite in soils with gas chromatography coupled to different detection systems, namely electron-capture and mass spectrometry. The methodologies developed for pyrethroids and 3-PBA analysis could be extremely relevant to further studies related with pyrethroids exposition and effects.

The spatial and seasonal occurrence of pyrethroid pesticides contamination in the environment was the focus of this work. No evidence was observed that support the presence of the pyrethroid pesticides analyzed in the Portuguese playground soil samples, considering that for the present limit of detection they were not found in the ten urban samples collected in the northern region of the country. Nevertheless, 17% of the agricultural soil samples tested had measurable levels for one target pyrethroid (deltamethrin) in the summer season with a maximum concentration level of 101.7 ng g^{-1}. A new, simple, rapid and robust analytical method for the determination in soils of a pyrethroid metabolite, 3-PBA, was developed based on aqueous solid-liquid extraction follow by SPE procedure and GC/MS detection. The proposed method was successfully applied to the analysis of eleven agricultural soils, showing the occurrence of 3-PBA in one soil sample at levels up to few ng g^{-1} of soil. Based on the developed methodology for 3-PBA and their subsequent detection in a real soil sample, besides pyrethroid pesticides their metabolites and transformation products have to be also included in monitoring studies.

Since the results herein present, deltamethrin soil contamination and the presence of 3-PBA in soils, deltamethrin was chosen as a monitoring example. The impact of deltamethrin on soil, after a commercial formulation application on Portuguese cabbage, was sensitively and effectively assessed. The deltamethrin levels over time in cabbage and soil as well as its metabolite, 3-PBA, in soil were monitored. The characterization of the soil microbial community over time was also assessed. The monitoring of deltamethrin in cabbage and soil had a similar decay in the first three days and disappeared completely after 180 days. The degradation half-life time ($T_{1/2}$) in

soil was equal to 8.8 days and degradation rate was 0.079 day^{-1}. Results clearly showed that, deltamethrin application caused an increase of the 3-PBA metabolite with a maximum concentration value of 23.2 ng g^{-1} after 15 days. This pyrethroid seemed to cause a shift in the microbial community structure of soil which was more noticed after 30 days. At this time, bacteria recognized to degrade pyrethroid and its metabolite such as *Nocardioides* sp. and *Sphingomonas* sp. had a significant increase. After 180 days of the application it was seen that the microbial community was still different from the initial one. So, deltamethrin, may cause an environmental impact much longer than the security interval. Although, more research is needed to better understand processes by which microorganisms potentiate natural degradation, the present study also shows that natural degradation can be an important technique for pesticides soil decontamination.

Toxicity tests are used for predicting the effects of chemicals on living biota, comparing sensitivity of one or more species to different chemicals, and setting rules for discharge regulation. The phytotoxic impact of pesticides was evaluated in *Cucumis sativus* (cucumber) seed. Some aspects such as germination, seedling development (root, leaf and shoot) and chlorophyll/carotenoid quantities were evaluated to understand the influence of these pyrethroids (cypermethrin, deltamethrin and cyhalothrin) and metabolite (3-PBA) on phytotoxicity. A direct effect of 3-PBA on germination was observed while the pyrethroids tested did not show any influence on this parameter. For the tested pyrethroids cypermethrin was the one with most impact on seedling development, namely on leaf and shoot elongation. Root elongation was more affected by deltamethrin. The impacts of the four compounds were also evaluated through the determination of chlorophyll and carotenoids contents after 7 days of exposure. Studies with deltamethrin were the ones that presented higher values for these two parameters with statistically significant differences for chlorophyll a, b, their ratio and for carotenoids. These results highlight the effect that pyrethroids have on the early growth of plants.

Overall, the present study confirmed pyrethroid pesticides and metabolite contamination in Portuguese soils and the need for their monitoring and assessment. A deeper knowledge of the environmental quality and the potential ecological impact of pyrethroid pesticides in soil is needed. While this study focuses in pyrethroid pesticides, the assessment of other compounds in soil could bring new important insights to the field. In the future perspective, work could be done considering the assessment of soil contamination in an extended territory, to better elucidate about possible effects on different levels of exposure, namely to a national/international level, both in urban and rural facilities.

Despite the contribution of the work here presented a gap of information stills exist considering Portuguese exposure to several environmental compounds to which the population is widely and daily exposed. Studies have shown that in the pesticides field, new trends of use and exposure are appearing. Some pesticides are being replaced for several new alternatives such as pyrethroids. Consequently, more studies, focused in the new alternative substances are needed. Little is known about the sources of exposure of these compounds to humans, particularly to children, as well as their possible health effects. On the other hand, the population and particularly children are exposed to a wide range of substances. Therefore, it is fundamental to consider the synergetic effect of the compounds.

Other test organism, at another trophic level, as *Daphnia magna* could be used to test pyrethroids toxicity. Preliminary studies shown that the pyrethroids tested (cypermethrin, cyhalothrin and deltamethrin) were found to be highly toxic as 48h EC50 were in the ppt (μg L^{-1}) range in acute toxicity tests according to the OECD guidelines.

Environmental monitoring is essential to evaluate contamination, for risk assessment and consequently for governmental institutions to perform the necessary actions for safety promotion.