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Ecotoxicological assessment of pesticide mixtures on terrestrial non-target species

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1 Introduction

SPRINT aims to provide tools and novel data to assess integrated risks and impacts of plant protection products (PPP; pesticides) on the environment and human health. Using this, SPRINT wants to accelerate the adoption of innovative transition pathways towards more sustainable plant protection.

A significant part of the SPRINT work package four (WP4) goals lies in the **ecotoxicity testing of pesticide mixtures on terrestrial organisms** (i.e. terrestrial non-target species). The focus of that effort is not only on standardized single-species low-tier assays with **EFSA test organisms** but also on **native species**, **new endpoints,** and **higher-tier** testing systems.

The testing concept chosen for the SPRINT project was based on **a whole-mixture approach** – i.e., organisms are exposed to the pesticide mixtures and the effects are evaluated (in contrast to the calculation approach based on single substances testing).

Selection of the pesticide mixtures was based on a **prioritization process** using the pesticide residue data based on the SPRINT survey 2021 performed at case study sites (CSS), substance properties, and hazard data (Jegede et al., 2024 - under review). See more in **Chapter 3**.

The ecotoxicological effects were tested **not only on current EFSA organisms and tests** but also on alternative (native) species and in multi-species systems, highlighting **indirect and chronic effects, bioaccumulation, and biomagnification, as well as better ecological relevance**. This way, **SPRINT proposes and validates a novel and/or improved strategy for testing the integrated effects of pesticide mixtures**. The overview of all tests planned is in the Description of Action (DoA), and more details were provided in Milestone 5 in December 2021. The overview of all tests performed during 2022 - 2024 is provided in **Table 1.1**.

Chapters 4 – 12 report methodologies, results, their discussion, and conclusions for individual tests performed. The last **Chapter 13** summarizes the significant findings, limitations, and outlook.

Looking at all the tests performed (**Table 1.1**), the SPRINT approach represented a **tiered approach**, from simple assessments at **low-tier standard single-species EFSA tests** to **"new SPRINT indicators"** using several alternative (native) species and to **higher-tier multi-species microcosms.**

Table1.1: Overview of all terrestrial ecotoxicology tests performed during 2022 – 2024. Table is organised similarly as Table 1.6 in DoA. For abbreviations of concentrations, see the next chapter. More details about the tests are provided in the following chapters as indicated in the last column.

Test	Endpoints	Duration	Number of pesticide mixtures tested	Concentrations tested	Number of replicates	Publication status (Aug 2024)	SPRINT partner mainly involved	Chapter
"EFSA tests"								
Soil microbial activity	C and N mineralization	28 (- 84 days)	11	MEC, PEC, 5PEC, CT, CTs	4	In preparation	MU	**4**
Collembola	Mortality, reproduction	28 days	11	MEC, PEC, 5PEC, CT, CTs	5	In preparation	MU	**5**
Earthworms	Mortality, growth, reproduction	28 and 56 days	11	MEC, PEC, 5PEC, CT, CTs	5	In preparation	WU	**6**
Beneficial insects	Mortality, reproduction	7 and 14 days	11	AR/2, AR, 2AR, CT, CTs, positive control	4	In preparation	WU	**7**
Terrestrial plants	Root growth, seedling emergence and early growth	4 and 14 days	11	MEC, PEC, 5PEC, CT, CTs	5	In preparation	MU	**8**
Honey bees	Acute: mortality Colony: mortality, food, brood	Acute: 48 hours Colony: 42 days	Acute: 11 Colony: 1	Acute: PEQ/4, PEQ/2, PEQ, 2.5PEQ, 5PEQ, 10PEQ, CT, CTs Colony: PEQ/4, 2.5PEQ, CT	Acute: 3 Colony: 5	In preparation	WU	**9**
"New SPRINT indicators"								
Soil microbiome - Functional and structural biodiversity	Phospholipid fatty acids Enzyme activities involved in C-, N- and P-cycles Functional genes based on qPCR Carbon use efficiency	28 and 56 days	4 11 + 2 11 + 4 2	Recommended field dose MEC, PEC, 5PEC, CT, CTs MEC, PEC, 5PEC, CT, CTs Recommended field dose	4 4 4 4	Under revision In preparation In preparation Under revision / In preparation	UH UH, MU, WU UH, MU, WU UH	**10**
Native earthworm species	Mortality, growth, reproduction for *L. rubellus* Mortality, cast production, burrowing activity for *L. terrestris*	28 and 74 days 74 days	11	MEC, PEC, 5PEC, CT, CTs	5	In preparation	WU	**6**
Soil microcosms	Earthworm survival and reproduction, plant growth, C and N mineralization	14, 28, and 56 days	1	MEC, PEC, 5PEC, CT, CTs	4	In preparation	MU	**11**
Wild bees	Colony mortality, food, brood	42 days	1	PEQ/4, 2.5PEQ, CT	10	In preparation	WU	**9**

2 General concept of the testing

The risk from exposure to chemical mixtures can be assessed by experimental testing of the mixtures, either present in real environmental matrices sampled or added to the matrices in the laboratory, (**whole-mixture approach**) or by mathematical predictions based on the individual components' toxicity results (**components-based approach**) (Bopp et al., 2018; More et al., 2019). In the SPRINT project, we followed a whole-mixture approach, and we tested the mixtures prepared in the laboratory and added them to the test organisms. **The measured effects in standard test species were compared to the component-based predictions** (for the known components of the mixture).

After the selection of the substances for each pesticide mixture (see **Chapter 3**), the mixtures were tested as follows:

At **5 concentrations** for soil invertebrates, microbial activities, enzymes and functional genes, terrestrial plants, and soil microcosms

- field concentration – measured environmental concentration (MEC)
- predicted exposure concentration from EFSA assessment (PEC)
- five times higher concentration than PEC (5PEC)
- control (CT)
- solvent control (CTs)

At **3 concentrations** for beneficial insects

- one half of the application rate (AR/2)
- application rate (AR)
- two times higher than the application rate (2AR)
- control (CT)

At **6 concentrations** for bees

- one-quarter of the predicted dietary exposure quantity (PEQ/4)
- half of the predicted dietary exposure quantity (PEQ/2)
- predicted dietary exposure quantity (PEQ)
- two and a half times the predicted dietary exposure quantity (2.5PEQ)
- five times higher than predicted dietary exposure quantity (5PEQ)
- ten times higher than predicted dietary exposure quantity (10PEQ)
- control (CT)

The field-recommended dose was used for novel soil indicators - phospholipid fatty acids and carbon use efficiency.

Most of the tests were performed with 3-5 replicates per concentration.

The concentrations of the pesticides added to the testing systems were usually verified by extractions and chemical analyses (**Chapter 12**). However, all the results are expressed in nominal concentrations, i.e., MEC, PEC, 5PEC, AR/2, AR, 2AR, PEQ, etc.

3 Selection of the pesticide mixtures for testing

In WP4, we developed a **prioritization approach** for selecting pesticides of concern found in environmental samples from the **case study sites (CSS)**. This prioritization aimed to select the most relevant pesticide mixtures in 11 CSS to be further assessed in ecotoxicological studies. The CSS were in Argentina (**AR**), Switzerland (**CH**), the Czech Republic (**CZ**), Denmark (**DK**), Spain (**ES**), France (**FR**), Croatia (**HR**), Italy (**IT**), the Netherlands (**NL**), Portugal (**PT**), and Slovenia (**SI**).

The pesticides in each CSS were ranked and scored according to their **detection frequency** in the soil samples and their **risk to soil organisms**. The risk was calculated as **risk quotient (RQ)**, a **ratio between exposure and toxicity**, with slightly different approaches for **4 groups of organisms**, namely soil invertebrates (earthworms and springtails), bees and beneficial insects, terrestrial plants, and soil microbes (**Figure 3.1**).

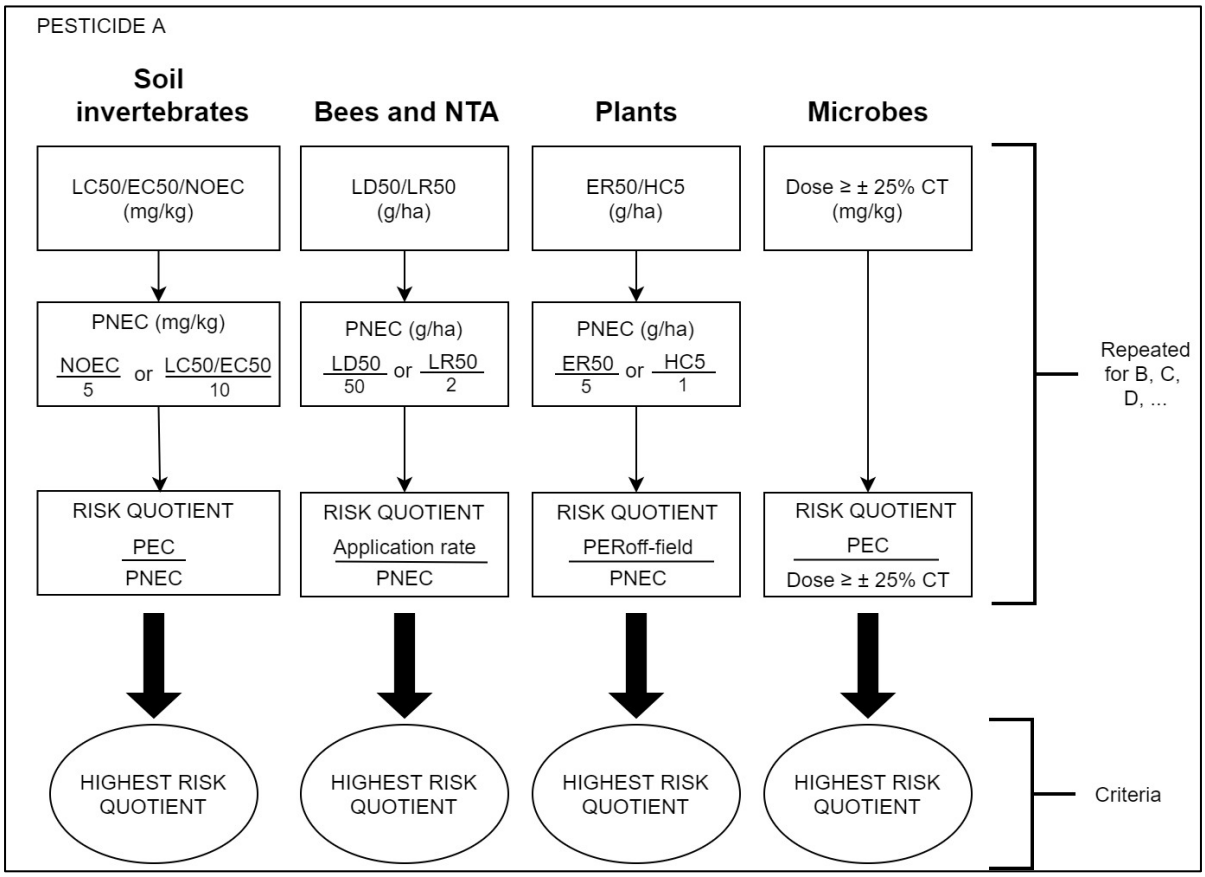

Figure 3.1: Risk calculation used as criteria for selecting the pesticide mixtures. The abbreviations are explained in the text.

For soil invertebrates, the values of NOEC (no observed effect concentration), LC50 (a concentration causing 50% lethality of the organisms), and EC50 (a concentration causing 50% effect on organisms compared to control) for standard species of soil invertebrates (*Eisenia fetida*, *Folsomia candida*, and *Hypoaspis aculeifer*) were retrieved from PPDB (http://sitem.herts.ac.uk/aeru/ppdb) and EFSA documents (i.e., conclusions and

assessment reports - http://www.efsa.europe.eu). Assessment factors for risk were applied to calculate the predicted no-effect concentration (PNEC). Assessment factors of 10 and 5 were used for LC50/EC50 and NOEC, respectively. The PNEC was derived by dividing the LC50/EC50 or NOEC by the assessment factors corresponding to the trigger values from the EFSA "toxicity vs. exposure ratio" (TER) approach. The risk quotient was calculated as PEC / PNEC.

For bees and non-target arthropods (NTAs), the dose-effect data were curated from PPDB (http://sitem.herts.ac.uk/aeru/ppdb) and EFSA documents (i.e., conclusions and assessment reports) (http://www.efsa.europe.eu). The LD50 (a dose causing 50% lethality) data for bees, *Apis mellifera*, *Bombus terrestris*, and mason bees, were used. For the NTAs, the LR50 data for the *Aphidius rhopalosiph*i and *Typhlodromus pyri* were used. The assessment factor of 50 was applied for the bee LD50 data, and the assessment factor of 2 was applied for the NTAs LR50 data to get PNEC. The exposure of the bees and NTAs was the application rate of the pesticides. These application rates were derived from the EFSA conclusions for each pesticide. The risk quotient was thus calculated as application rate / PNEC.

For terrestrial plants, ecotoxicological data (ER50 or HC5) were extracted from EFSA documents (i.e., conclusions and assessment reports) (http://www.efsa.europe.eu), except for chlortoluron which data was obtained from the literature (Song et al., 2010). ER50 is a rate causing 50% effect compared to control and HC5 is a concentration causing significant effects on 5% of species. Assessment factor values of 5 and 1 were used to adjust ER50 and HC5 values, respectively. PNEC was calculated as ER50 or HC5 divided by assessment factors values. The exposure of terrestrial plants was derived as the highest predicted environmental rate (PERoff-field) also collected from EFSA. If PERoff-field was not provided, the application rate was used. The risk quotient was calculated as exposure (PERoff-field or application rate) / PNEC. The same unit was used for exposure and PNEC in the calculation (either as g/ha, mL/ha, or mg/kg).

For microbes, the effects of pesticides on soil nitrogen transformations were the endpoint used, because data on other endpoints (carbon transformation, enzymatic activities) were less available. The data was extracted from the EFSA conclusions (http://www.efsa.europe.eu). The concentration showing ≥25% deviation from control for effects on nitrogen mineralization was used as the dose effect for calculating the risk quotient. No assessment factor was applied for microbes since only one endpoint was considered.

For each organism group, **scores for „occurrence in soils" and „risk quotient"** were obtained in each CSS. Occurrence in soil was calculated as the percentage of detection (detection frequency) in the soil samples in the CSS. The risk quotient was divided by the highest score obtained in each CSS, to normalize all scores to 1. The **normalized scores were multiplied**, and the pesticides were ranked. The top 5 pesticides with the highest scores were selected in each CSS.

A relevant **mixture of up to 5 pesticides** was chosen, as most agricultural soils contain 2 to 10 pesticide residues (Silva et al., 2019; Geissen et al., 2021; Kosubová et al., 2020). Moreover, according to the EU Commission on assessment of mixtures, many studies have reported that few pesticides found in mixtures drive the most toxicity potency of the respective mixtures (EC, 2020). Therefore, ecotoxicological tests do not need to be carried out with all the pesticides in the mixtures but should instead primarily focus on **priority pesticides that drive the overall toxicity of the mixture**.

In the **Appendix**, the final selection of the mixtures is displayed in **Tables A1 - A4**.

Of the 192 substances analysed in soils in WP2, 55 were included in the final prioritization list, considering all groups of organisms. These mixtures were used in the terrestrial toxicity tests. The mixture selection was composed of the same pesticides in ES and IT for soil invertebrates and microbes. Therefore, 11 mixtures were tested for bees and NTAs and terrestrial plants, and 10 mixtures were tested for soil invertebrates and microbes. In total, **42 different mixtures of pesticides were tested**.

4 Soil microbial activity

4.1 Introduction

Soil microorganisms encompass all organisms smaller than 200 µm, composed mainly of bacteria and fungi but also algae, protozoa, and nematodes (Joergensen & Wichern, 2018). Microbial activities play an essential role in the decomposition of organic matter and cycling of nutrients and, therefore, are good bioindicators of the health status of soils. In agricultural soils, microorganisms are intrinsically related to soil fertility and are also involved in symbiotic relationships with crop plants and the degradation of contaminants, such as pesticides.

Carbon and nitrogen mineralization have been implemented in standard tests to assess the effects of chemicals and have also been adopted in the ecological risk assessment of active ingredients and PPP (EC 2013a; EC 2013b). In the carbon mineralization tests, CO_2 production is measured in untreated soil (basal respiration) or glucose-amended soil (substrate-induced respiration). For the nitrogen mineralization tests, the levels of nitrogen forms related to the nitrogen cycle (i.e., ammonium, nitrite, and/or nitrate) are measured in soils after the provision of a nitrogen source.

Pesticide exposure can either stimulate microbial activity or suppress it. It has been established that the activity in pesticide-treated soils cannot overcome the critical level of ±25% of the control. If the treated soil presents deviations from the control >±25%, the experiments must be extended up to 100 days (ISO, 2002; ISO, 2012a; OECD, 2000a; OECD 2000b). Although all active substances and PPP are expected to cause no deleterious effects at PEC, the potential effects of mixtures containing multiple pesticides on microbial activities have been poorly addressed.

4.2 Methodology

The microbial activity was assessed as carbon and nitrogen mineralization. The **basal respiration** (BR) and **substrate-induced respiration** (SIR) methods were adapted from ISO guidelines 16072 (ISO, 2002) and OECD 217 (OECD, 2000b), respectively. The **potential ammonium oxidation** (PAO) and **potential ammonification** (PAMO) were measured according to ISO guideline 15685 (ISO, 2012a) and Alef (1995). Before the spiking, the soil was amended with powdered lucerne meal (0.5%) and pre-incubated for 2 weeks to activate the activity of the microbes. The soil was then spiked at 3 concentrations, namely MEC, PEC, and 5PEC using acetone solvent as a carrier. The negative control and solvent control were tested, too. Four replicate test jars were prepared for each concentration. The test jars were incubated at 22 °C in the dark. After 28 days of incubation, the measurements of microbial activity were conducted. For BR, bottles were sealed with a rubber lid and kept at 22 °C in the dark, and CO_2 was measured after 24h by gas chromatograph (GC, Agilent Technologies 6850). For SIR, glucose solution (5 g per kg of soil) was added while adjusting the soil moisture to 80% WHC. The CO_2 production was measured after 2.5 and 5h by GC. For PAMO and PAO, KCl was added

to the incubated samples and analysed by flow injection (FIAlab 2500) for ammonium and nitrite ions, respectively. In the treatments where the deviation from negative and/or solvent control was ≥±25%, the measurements were repeated in 28-day intervals (i.e., 56 and 84 days).

4.3 Results & Discussion

The differences between the negative and solvent controls were within 22%, indicating that **no significant effect was caused by adding the solvent** (acetone).

The results showed that deviation from the solvent controls was higher than ±25% for at least one endpoint in **6 out of the 11 CSS** (**Table 4.1**). The 5 CSS, where no effect was observed after 28 days of incubation, were DK, SI, HR, CH, and AR.

Effects on **carbon mineralization** occurred at 5PEC in the CZ, ES/IT, FR, and PT mixtures. After 56 days, no effects were longer observed, except in the PT mixture, where deviation from control decreased to <25% only after 84 days. It was also observed that the mixtures from PT and FR stimulated the CO_2 production in contrast to the inhibition of the CO_2 production in the mixtures from ES/IT and CZ. The mixtures showing effects on basal respiration (CZ, ES/IT, PT) were the most similar from all tested mixtures, having 3 pesticides in common: the fungicides boscalid, tebuconazole, and metrafenone. The mixture from FR, which was the only one showing effects on substrate-induced respiration, also had the fungicides boscalid and metrafenone in its composition. One mixture (HR) contained boscalid and tebuconazole, however no effect on carbon mineralization was observed.

Table 4.1: List of case-study sites (CSS) with ≥±25% deviation from the solvent control after 28, 56, or 84 days of exposure for the endpoints of carbon mineralization (Cmin) and nitrogen mineralization (Nmin).

	CSS	Endpoint	Concentration	28d	56d	84d
Cmin	CZ	BR	5PEC	-29%	-2%	
	ES/IT	BR	5PEC	-23%*	-1%	
	FR	SIR	5PEC	32%	0%	
	PT	BR	5PEC	27%	35%	19%
Nmin	CZ	PAMO	PEC	-38%	28%	-2%
	CZ	PAMO	5PEC	-32%	37%	-20%
	CZ	PAO	PEC	-26%	-8%	
	CZ	PAO	5PEC	-27%	-1%	
	NL	PAMO	PEC	-44%	-2%	
	NL	PAMO	5PEC	-27%	22%	

*CZ: Czech Republic; ES/IT: Spain and Italy; FR: France; NL: The Netherlands; PT: Portugal; BR: basal respiration; SIR: substrate-induced respiration; PAMO: potential ammonification; PAO: potential ammonium oxidation; 5PEC: five times 'predicted environmental concentration'. * Deviation from negative control of -33%.*

Nitrogen mineralization was affected by the mixtures from CZ and NL after 28 days at PEC and 5PEC. In all cases, the microbial activity decreased. The deviation from control

was <25% after 56 days in the NL mixture and only after 84 days in the CZ mixture. These mixtures only had the fungicide boscalid in common. Another similarity between both mixtures, exclusive from these 2 CSS, was the presence of strobilurin fungicides, dimoxystrobin and fluoxastrobin.

Although the effects on C and N mineralization persisted up to 56 days in the most toxic CSS (PT), the microorganisms recovered after 84 days.

4.4 Conclusions

- The effects on carbon mineralization could be related to the fungicides metrafenone, tebuconazole, and boscalid, as a mixture.
- PAMO and PAO were more sensitive endpoints than BR and SIR, with effects at PEC.
- The mixture from PT was the most toxic for carbon mineralization, while the mixture from CZ was the most toxic for nitrogen mineralization. In both mixtures, the microbial activity was restored after 84 days.

5 Collembola

5.1 Introduction

Collembolla (springtails) are soil-dwelling organisms with great ecological relevance for the soil ecosystem and are one of the most abundant groups of the soil mesofauna (Hopkin, 1997). Springtails are crucial in various soil processes, including the carbon and nitrogen cycles (Filser, 2002). They also have high ecological importance due to their interactions with other soil biota, such as plants and microorganisms. *Folsomia candida* is a parthenogenetic species and has a high rate of reproduction. This species is easy to culture under laboratory conditions. Therefore, *F. candida* has been widely used as a standard model organism in soil ecotoxicology studies. The reproduction test with *F. candida* is a standardized ecotoxicological test (OECD, 2009; ISO, 2014) that has been vastly applied to assess the effects of pesticides. Moreover, the reproduction test with *F. candida* is used as a requirement for the approval of active substances and authorization of PPP within the EU legislation (EC, 2013a; EC, 2013b). Several studies have shown that *F. candida* is among the most sensitive organisms to pesticide exposure (Joimel et al., 2022; Panico et al., 2022). Considering the factors mentioned above, ecotoxicological studies with *F. candida* are an essential part of the risk assessment of pesticide mixtures.

5.2 Methodology

A reproduction test with the springtail ***Folsomia candida*** was conducted following the OECD 232 guideline (OECD, 2009). The springtail *Folsomia candida* was cultured in transparent plastic boxes with a moist bottom of plaster of Paris and charcoal at 20 °C. The organisms were fed with dry yeast (*Saccharomyces cerevisiae*) once a week. The tests were conducted in glass jars containing approximately 25 g of moist soil. Five replicates per concentration were prepared, and one extra replicate was prepared (no animal added) for the **chemical analyses**. For each mixture, 3 concentrations were used: MEC, PEC, and 5PEC. Negative (CT) and solvent (CTs) controls were also conducted. At the start of the test, 10 springtails (10-12 days old) were transferred into each jar, and dry yeast was added as food. Test jars were incubated in a climate room at 20 °C and a photoperiod of 16 h: 8 h (light: dark). Water loss was replenished weekly, and food was added after 2 weeks. After 4 weeks, the test was ended by adding tap water into the test jars. The content was transferred into a plastic container and stirred carefully to let all the springtails float on the surface. Photographs were taken with a digital camera, and the number of **surviving adults and juveniles** was counted using ImageJ software (version 1.53k). A one-way ANOVA test was conducted after checking data normality and homoscedasticity.

5.3 Results & Discussion

Springtail survival significantly decreased at PEC and 5PEC concentrations in the FR and PT mixtures (**Figure 5.1**). A significant decrease in **reproduction** was observed in springtails exposed to PEC and 5PEC treatment in the ES, FR, IT, and PT mixtures, and

5PEC treatment in the HR mixture. The toxicity observed in the FR and PT mixtures was higher than expected when considering the NOEC values of the single pesticides reported by EFSA. The mixture composition of most toxic CSS (ES/IT, FR, PT) had the insecticide chlorantraniliprole, which is highly toxic to springtails, and the fungicides boscalid and difenoconazole. Azole fungicides, such as difenoconazole, are known to inhibit the metabolization of insecticides, causing synergetic effects (Cedergreen, 2014). It was observed that the synergetic effect on honeybees from the co-exposure of chlorantraniliprole with difenoconazole was caused by the inhibition of cytochrome P450 monooxygenases, which are involved with the detoxification of chlorantraniliprole (Haas et al., 2022).

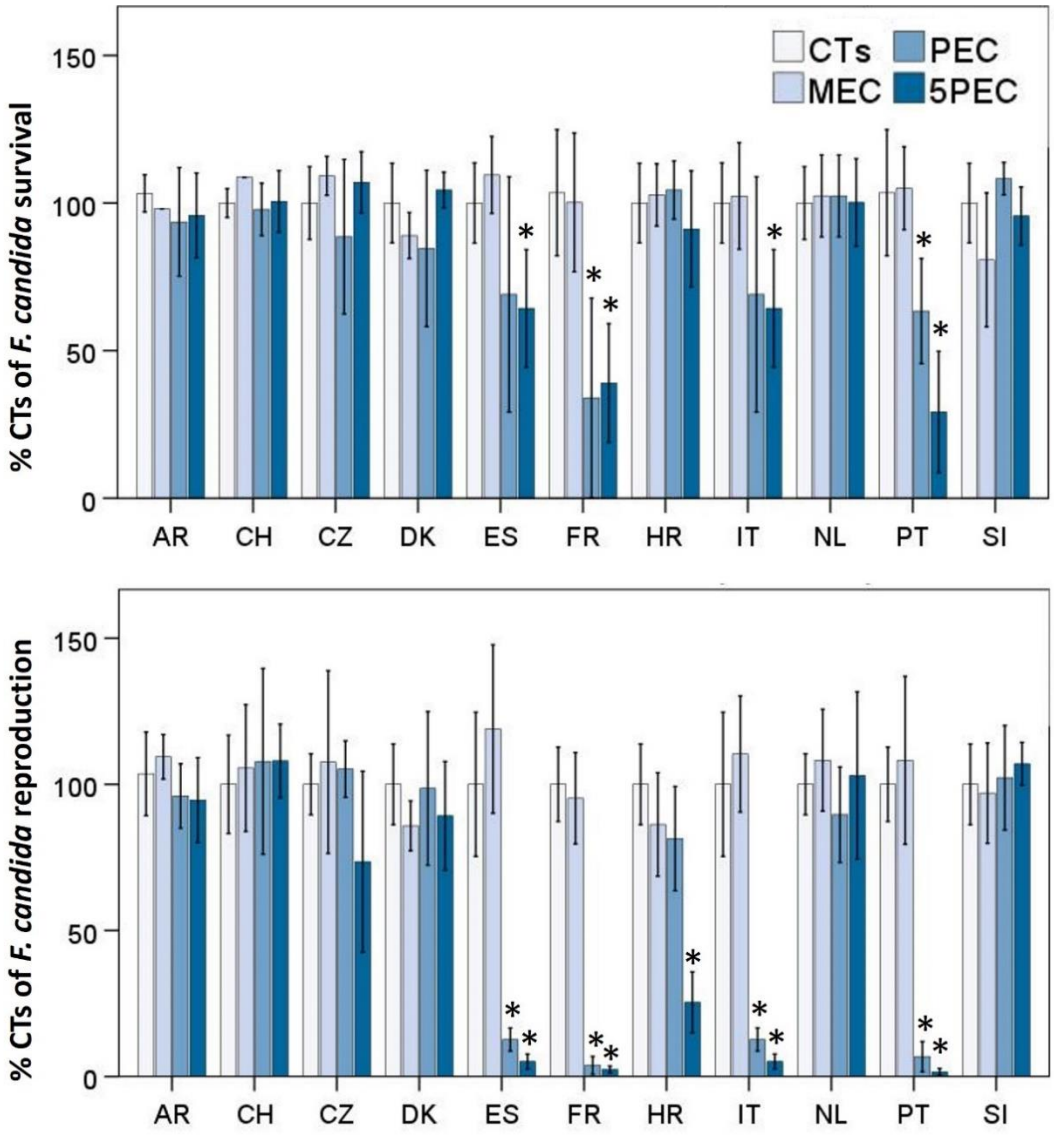

Figure 5.1: Effects of pesticide mixtures on springtail survival (top) and reproduction (bottom). Results (mean ±SD) are expressed as the percentage of the solvent control (CTs). Asterisks indicate significant differences compared to solvent control (Dunnet's post hoc test, $p<0.05$). AR: Argentina; CH: Switzerland; CZ: Czech Republic; DK: Denmark; ES: Spain; FR: France; HR: Croatia; IT: Italy; NL: The Netherlands; PT: Portugal; SI: Slovenia.

5.4 Conclusions

- Reproduction was the most sensitive endpoint, with a significant decrease observed in 5 CSS (ES/IT, PT, FR, HR) at both PEC and 5PEC level.
- Survival was significantly affected in 4 CSS (ES/IT, PT, FR) at both PEC and 5PEC levels.
- Most effects were observed from PEC exposure, while no effects were observed in springtails exposed at MEC.
- The insecticide chlorantraniliprole was present in the most toxic mixtures and probably drove the toxicity of the mixtures.

6 Earthworms

6.1 Introduction

Earthworms are essential to soil organisms involved in organic matter degradation and nutrient cycling, and their burrowing helps with soil aeration (Liu et al., 2019). Due to their essential activities on / in the soil surface, they are usually exposed to contaminants such as pesticides. As the first soil organism adopted for assessing the toxicity of chemicals in soil, Eisenia fetida/andrei is considered a standard species widely used in soil toxicity testing and a standard EFSA organism (Van Gestel, 2012). *Eisenia fetida/andrei* is an epigeic worm adopted as a standard species because of its ease of culturing in the lab and reproducibility in tests. However, *E. fetida/andrei* are not considered true in-soil organisms because they are compost worms (Pelosi et al., 2013). Therefore, compared to true soil species, *E. fetida* may not be as exposed to pesticides applied in agroecosystems.

Lumbricus rubellus and *Lumbricus terrestris* are epigeic and anecic earthworms, respectively, which are true soil species native to Europe (Klein et al., 2020). *L. rubellus* and *L. terrestris* are expected to be truly exposed to pesticides applied in agroecosystems. Despite many studies on the effect of pesticides on earthworms, toxicity data has been relatively scarce for these two native species compared to the standard earthworm species. From the few studies used in a meta-analysis, *L. rubellus* was reported to be less sensitive to pesticides than *E. fetida,* while *L. terrestris* was reported to be more sensitive to *E. fetida* (Pelosi et al., 2013). It is essential to use native soil species for pesticide toxicity tests in European soils in addition to standard test species because they provide realistic and relevant data for terrestrial environmental risk assessment of pesticides.

6.2 Methodology

Adult and clitellated earthworm species were used for this toxicity test. Three separate tests were conducted for each of the earthworms. The test with ***E. fetida*** followed the standard OECD 222 procedures (OECD, 2016). For the epigeic ***L. rubellus***, the test also followed the OECD 222 procedures but was slightly modified by making the test duration 74 days instead of 56 days for *E. fetida*. The anecic ***L. terrestris*** did not follow the OECD 222 procedures because of its large size, behaviour and niche. *L. terrestris* was exposed in glass mesocosms (**Figure 6.1**), as described by Huerta Lwanga et al. (2017).

The earthworms were exposed to three concentrations of 11 pesticide mixtures for these tests: MEC, PEC, and 5PEC. The water and the solvent control were also tested. The test concentrations and controls were done in five replicates. Each replicate was made up of 500 g of soil dosed with a mixture of five pesticide active substances. All the pesticides were dosed and mixed into the soil using acetone as solvent, except glyphosate and AMPA, which were dosed and mixed in the soil with water. To dose the soils, the pesticide-solvent was added to 10% w/w of the soil, and the soils were exposed in the fume hood for 24 hours to allow for the total evaporation of the acetone and ensure that only the active substances were in the soil. Then the rest of the 90% w/w of the soils were added and the

soils were moistened with water at 50% WHC and then put in the jars for the test (**Figure 6.2a**). The **growth and survival** of *E. fetida* and *L. rubellus* were assessed after 28 days by taking the adults out of the soil, counting and weighing them. At this time, cocoons were expected to have been laid in the soil. Then, the **juveniles** were extracted using a water-bath (**Figure 6.2b**) after another 28 days for *E. fetida* and 46 days for *L. rubellus*, making a total duration of 56 days for *E. fetida* and 74 days for *L. rubellus*.

However, for *L. terrestris*, the glass mesocosms were opened on the 74th day and number of surviving adults were assessed as **adult survival**, the weight of the surviving adults was assessed as **growth**, the cast biomass produced on the soil surface was assessed as **cast production, and** the length and width of the tunnels made by the earthworms were calculated **as tunnel volume,** which is a proxy for burrowing capacity after 74 days of testing. Tunnels were formed as illustrated here (**Figure 6.1**).

(a) (b)

Figure 6.1: (a) The *L. terrestris* added into the soil in the glass mesocosm test (b) the view of the *L. terrestris* making burrows and tunnels in the soil in glass mesocosm after 74 days of test.

(a) (b)

Figure 6.2: Set-up of the earthworms (*E. Fetida* and *L. rubellus*) experiments in the laboratory: (a) earthworms already in the dosed soil and is incubating during tests (b) juvenile extraction of earthworms after 56 days for *E. fetida* and 74 days for *L. rubellus*.

One-way analysis of variance (ANOVA) was used to test the effect of the concentration on the earthworm survival, growth, and reproduction. The Tukey post hoc comparison was

used to test where there are significant ($p<0.05$) differences. Where toxic effects were observed, the individual pesticide concentrations were compared with the NOEC of the pesticide based on the standard species.

6.3 Results & Discussion

6.3.1 Eisenia fetida

Pesticide mixtures from five CSS (AR, HR, SI, CZ, and PT) were toxic to *E. fetida* (**Figure 6.3**). Growth and reproduction were the more sensitive endpoints compared to survival. Sublethal endpoints have been reported to be more sensitive than lethal endpoints (Gunstone et al., 2021). There was total reproduction inhibition from PEC for CZ mixture and 5PEC for HR, making the CZ mixture the most toxic to *E. fetida*. At least one of the pesticides in the mixtures was at the concentration (5PEC) above the NOEC, for four of the CSS except the SI mixture (**Figure 6.4**). Therefore, it is expected that there may be some toxic effects from these 4 mixtures because at least one of the pesticides' concentrations is higher than NOEC at 5PEC. For example, the CZ mixture contains thiophanate methyl, which was orders of magnitude above NOEC at PEC and 5PEC. However, in the SI mixture, all the individual pesticides of the mixture were at concentrations below the NOEC, even at 5PEC. Therefore, the effect posed by the SI mixture on the earthworm is either additive or synergistic.

6.3.2 *Lumbricus rubellus*

Pesticide mixtures from 5 CSS (DK, CH, NL, HR and CZ) were toxic to *L. rubellus* (**Figure 6.5**). Reproduction is the most sensitive endpoint for *L. rubellus* with 100% inhibition at 5PEC in the CZ mixture. CZ mixture is the most toxic to *L. rubellus* out of the 5 mixtures. Because in a literature review, we could not find single pesticide toxicity data for *L. rubellus* (Jegede et al., in prep.), we could not ascertain if single pesticide concentrations were above the NOEC. However, being an epigeic species as well, the NOEC for the stand species was used for the comparison to the individual pesticide concentrations of the mixture. The result showed that there were possibilities of mixture effect in the DK and NL mixtures (**Figure 6.6**). In the DK mixture, despite all individual pesticide concentration below NOEC, reproduction was significantly affected at PEC. The effect on reproduction tends to be consistent in 5PEC but not significantly. In the NL mixture, significant effect on reproduction appeared in MEC, despite that the individual NOECs were not exceeded. It is important to note that there was no NOEC value for prosulfocarb in the NL mixture, so the literature LC50 value for E. fetida was used. The effect on reproduction was again significant at 5PEC but boscalid 5PEC was slightly higher than its NOEC value. For SW and CZ mixtures, at least one pesticide concentration was higher than the NOEC at the point of significant effect. In the SW mixture, survival was affected at 5PEC where difenoconazole concentration was higher than its NOEC but the other four were lower. For the CZ mixture, thiophanate methyl concentrations at PEC and 5PEC were higher than NOEC, so the mixture toxic effect may be driven by this pesticide.

6.3.3 Lumbricus terrestris

For *L. terrestris*, only two mixtures (CZ and PT) were toxic (**Figure 6.7**). CZ mixture caused 100% mortality of *L. terrestris* at 5PEC, and every other endpoint was impacted (growth, tunnel volume, and cast biomass). PT mixture impacted cast biomass produced by *L. terrestris* at 5PEC. Cast biomass was more sensitive as a sublethal endpoint based on the number of mixtures that impacted it. Being an anecic species with a different experimental design and mostly different endpoints from the standard species, we did not compare the individual pesticides concentrations with the NOEC values.

6.3.4 Pesticide mixture risk detection

Pesticide mixture risk on the earthworms were not detected in some of the case study sites (CSS) but for the other sites, risk was detected based on at least one earthworm. This study showed that the pesticide mixture risks detected in five CSS (AR, HR, SI, CZ, PT) for the standard species *E. fetida.* When the risk detected for the native species were incorporated with the standard species, the total detected mixture risk or CSS was eight i.e., AR, HR, SI, CZ, PT, DK, CH, NL (**Table 6.1**). Using more species for tests is desirable to provide more data for environmental protection (Lee et al., 2024).

Table 6.1. Pesticide mixtures effect on the three earthworms, *Eisenia fetida* (Standard species), *Lumbricus rubellus* and *Lumbricus terrestris* (native species) based on case study sites. The mixtures identified by red text were the mixtures that caused a significant effect on at least one earthworm species measured based on at least one endpoint

Earthworms	Pesticide mixtures represented by Case study sites										
Eisenia fetida	**AR**	CH	**CZ**	DK	ES	FR	**HR**	IT	NL	**PT**	**SI**
Lumbricus rubellus	AR	**CH**	**CZ**	**DK**	ES	FR	HR	IT	**NL**	PT	SI
Lumbricus terrestris	AR	CH	**CZ**	DK	ES	FR	HR	IT	NL	**PT**	SI
At least one species was affected by a mixture	**AR**	**CH**	**CZ**	**DK**			**HR**		**NL**	**PT**	**SI**

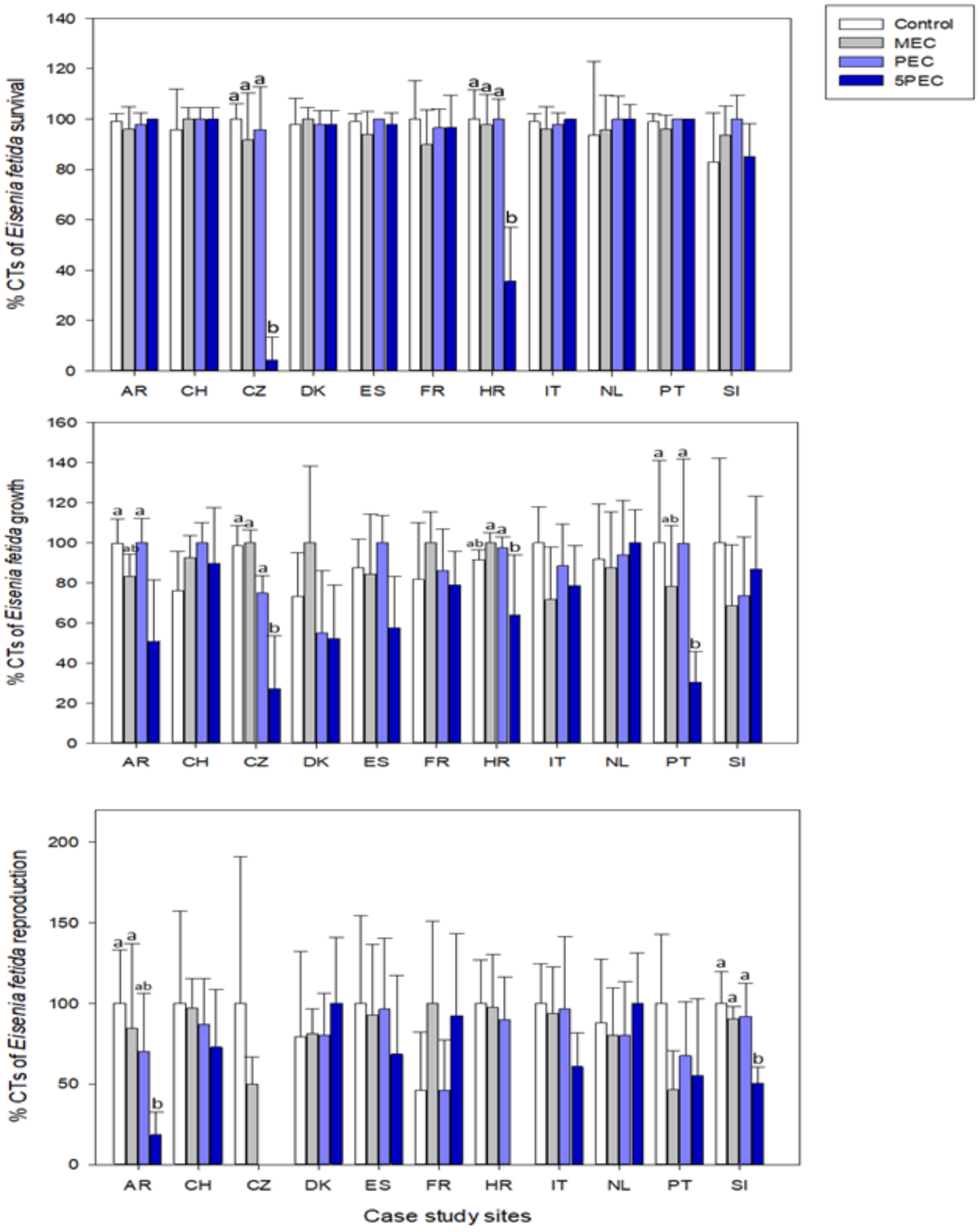

Figure 6.3: The effects of pesticide mixtures based on case study sites on the survival (upper panel), growth (middle panel) and reproduction (lower panel) of *Eisenia fetida*. Results (mean ± SD) are expressed as the percentage of control (CTs). AR – Argentina, CH – Switzerland, CZ – Czech Republic, DK – Denmark, ES - Spain, FR – France, HR – Croatia, IT – Italy, NL – Netherlands, PT – Portugal, SI - Slovenia. The letters "a, b, c" represents significant differences ($p < 0.05$) and bars with same letters represent no significant difference ($p > 0.05$).

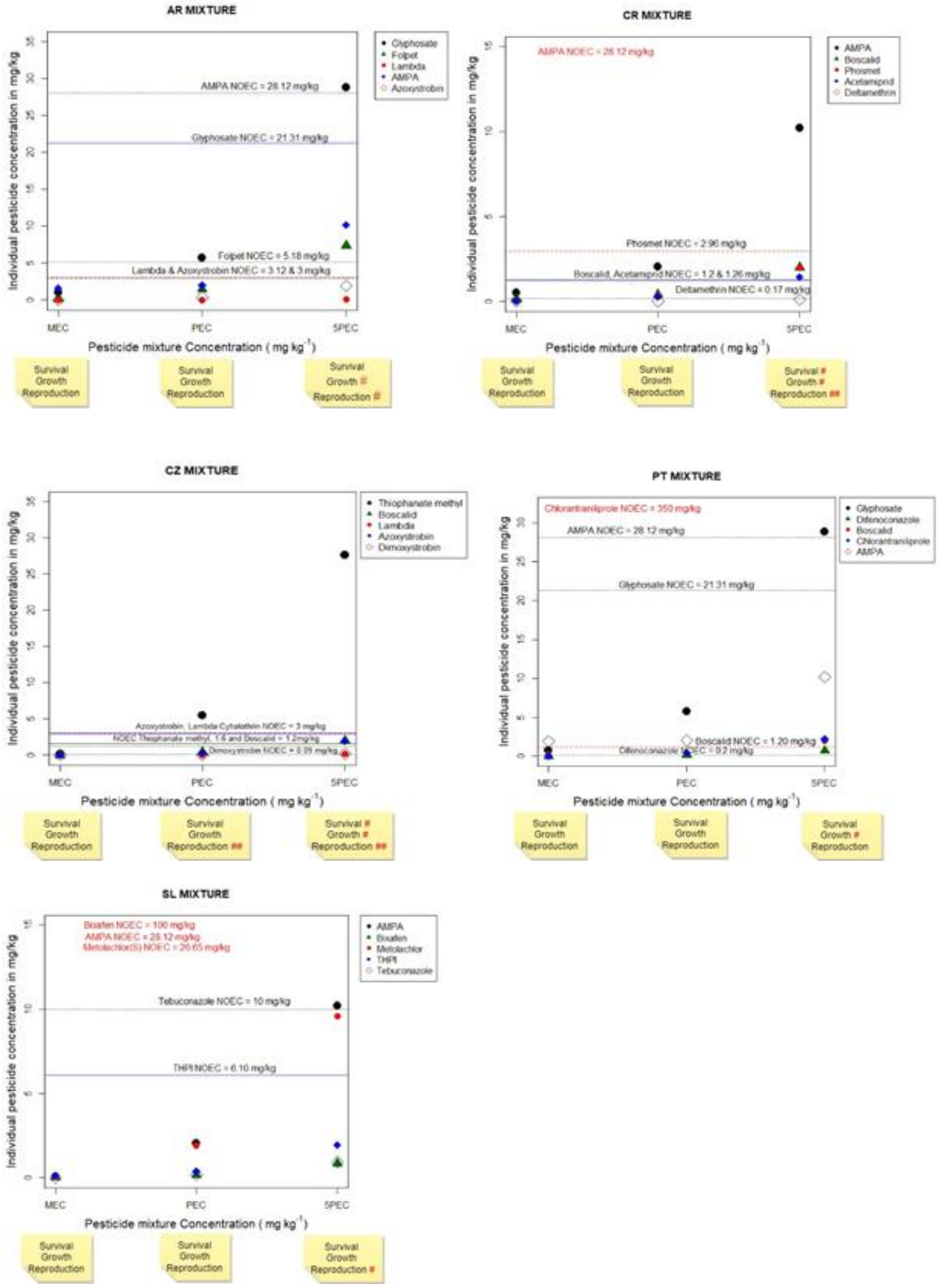

Figure 6.4: Single pesticide NOEC values of *E. fetida* and their corresponding measured environmental concentration (MEC), predicted environmental concentration (PEC) and 5PEC in relation to the toxicity endpoints (survival, growth, reproduction of *E. fetida*) in the pesticide mixture tests. The MEC, PEC and 5PEC are denoted by the horizontal lines. The letters in red are NOEC values above any of the concentrations. # = significant difference ($p<0.05$), ## = 100% effect

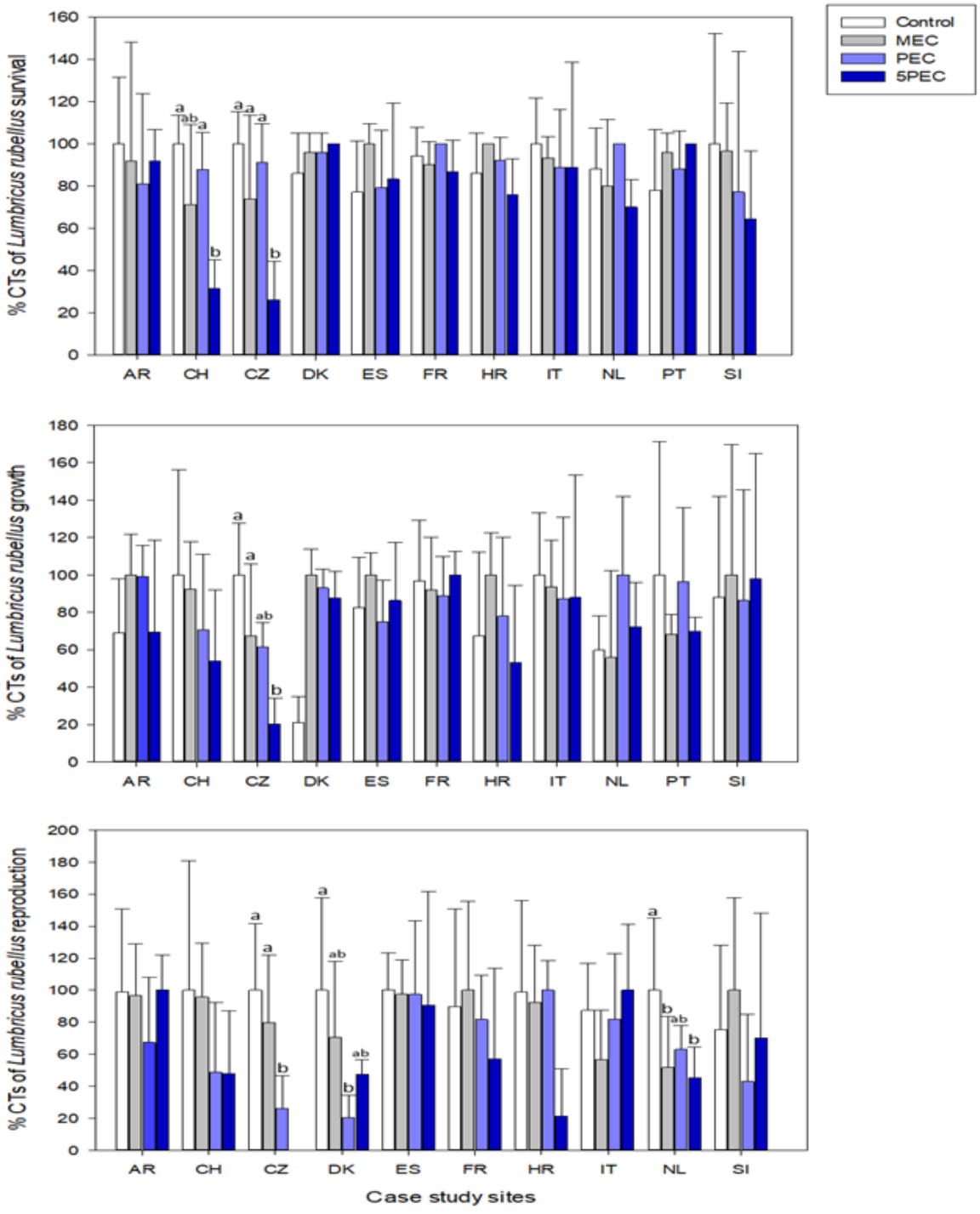

Figure 6.5 The effects of pesticide mixtures based on case study sites on the survival (upper panel), growth (middle panel) and reproduction (lower panel) of *Lumbricus rubellus*. Results (mean ± SD) are expressed as the percentage of control (CTs). AR – Argentina, CH – Switzerland, CZ – Czech Republic, DK – Denmark, ES - Spain, FR – France, HR – Croatia, IT – Italy, NL – Netherlands, PT – Portugal, SI - Slovenia. The letters "a, b, c" represents significant differences ($p < 0.05$) and bars with same letters represent no significant difference ($p > 0.05$).

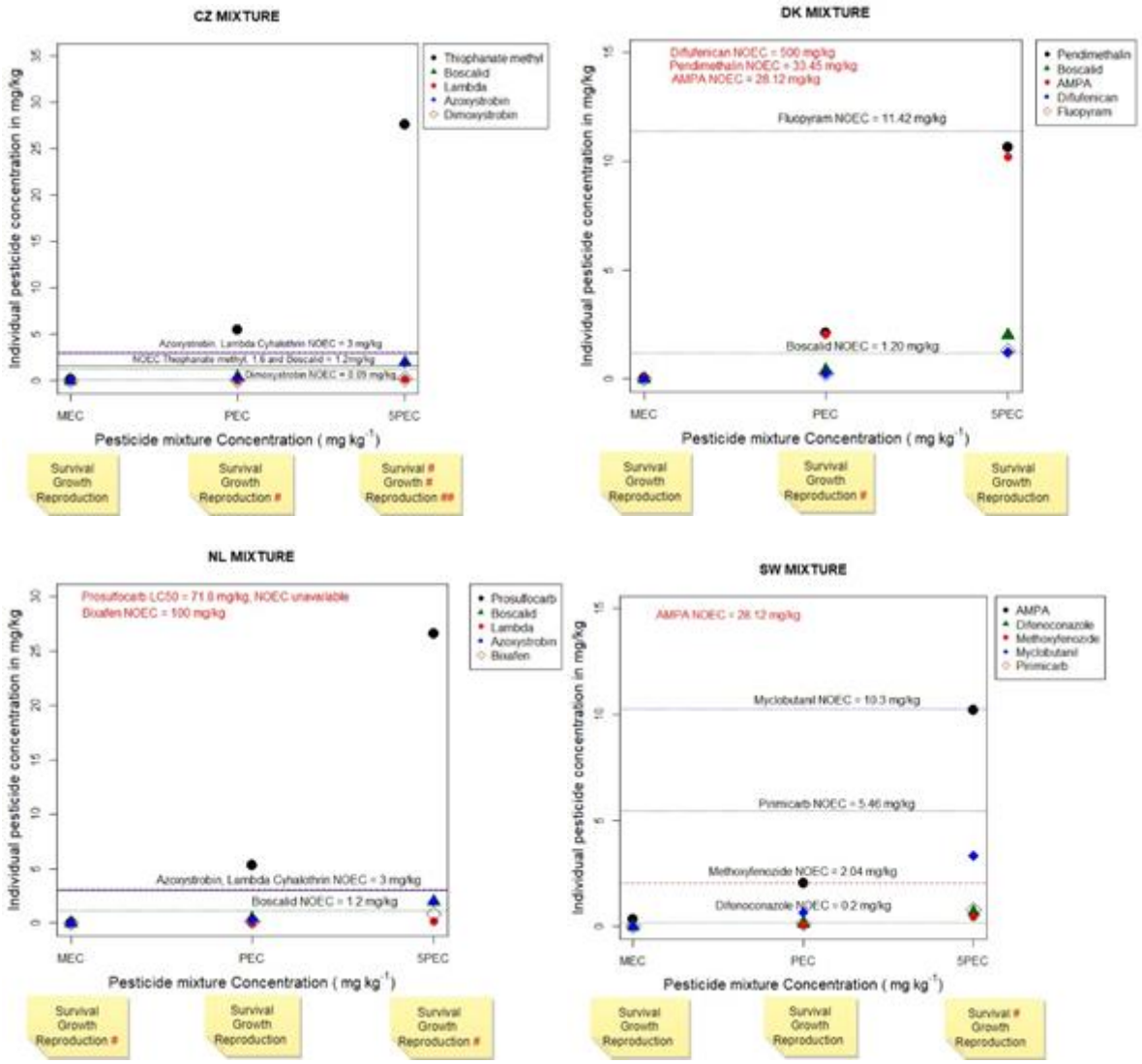

Figure 6.6: Single pesticide NOEC or LC50 values of E. fetida and their corresponding measured environmental concentration (MEC), predicted environmental concentration (PEC) and 5PEC in relation to the toxicity endpoints (survival, growth and reproduction of L. rubellus) in the pesticide mixture tests. The MEC, PEC and 5PEC are denoted by the horizontal lines. The letters in red are NOEC/LC50 values above any of the concentrations. # = significant difference ($p < 0.05$), ## = 100% effect.

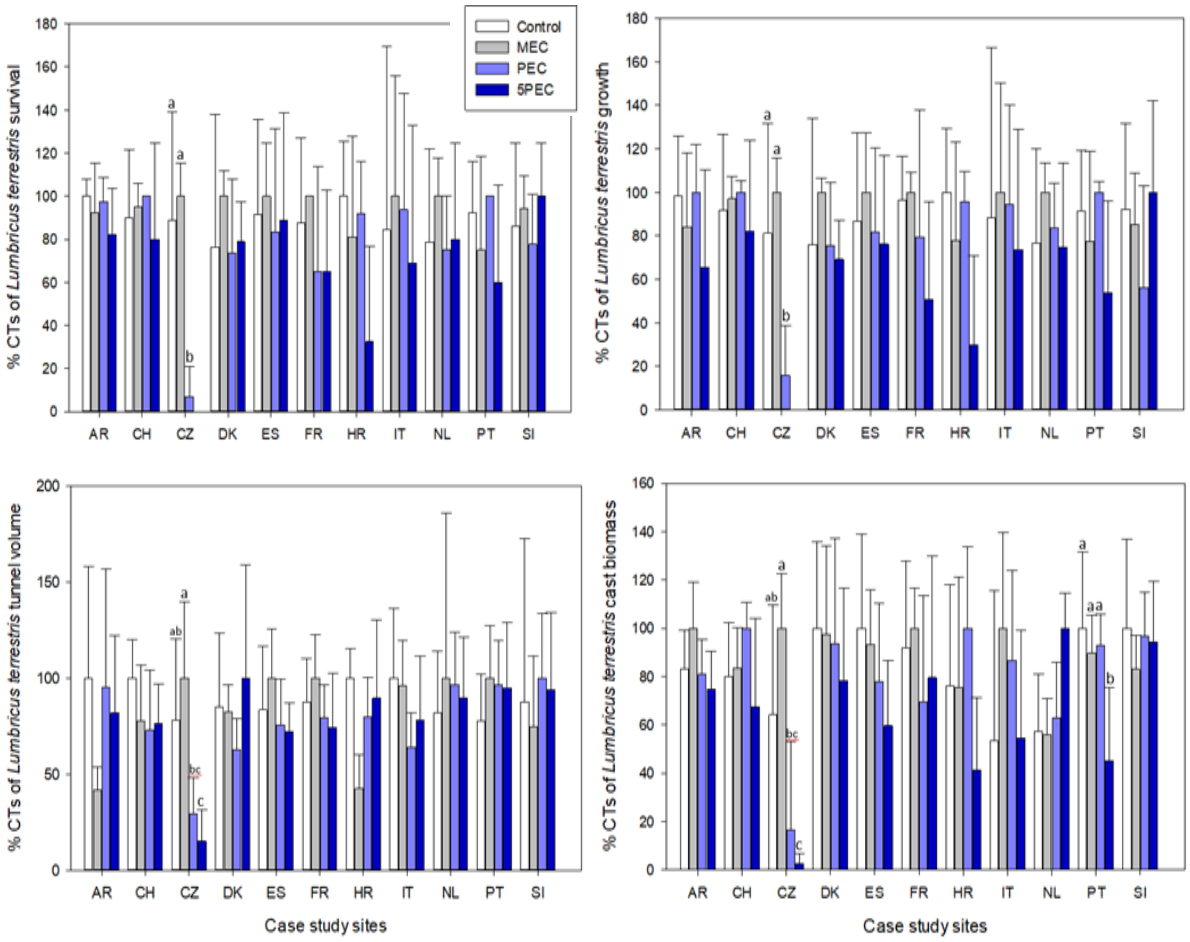

Figure 6.7: The toxic effects of pesticide mixtures based on case study sites on the survival (upper left panel), growth (upper right panel), tunnel volume (lower left panel) and cast biomass (lower right panel) produced by *Lumbricus terrestris*. Results (mean ± SD) are expressed as the percentage of control (CTs). AR – Argentina, CH – Switzerland, CZ – Czech Republic, DK – Denmark, ES - Spain, FR – France, HR – Croatia, IT – Italy, NL – Netherlands, PT – Portugal, SI - Slovenia. The letters "a, b, c" represents significant differences ($p < 0.05$) and bars with same letters represent no significant difference ($p > 0.05$).

6.4 Conclusions

- For the two epigeic worms, CZ and HR mixtures were consistently the most toxic.
- CZ mixture was the most toxic to the earthworms, regardless of their ecological type.
- Sublethal endpoints were more sensitive than the lethal endpoint for all the earthworms.
- Mixture toxicity effects were observed, especially for SL, DK and NL mixture where the concentrations of all their individual pesticide components were below NOEC. Therefore, risk assessment based on single pesticide toxicity data may not be protective.
- Using the standard species, only five mixtures showed risks (AR, HR, SI, CZ, PT). However, using the native species, the additional mixtures (DK, CH, and NL) revealed toxic effects.

7 Beneficial insects

7.1 Introduction

Testing on the effects of pesticide mixture residues on beneficial non-target arthropods focused on a predatory mite species *Amblyseius swirskii (Acari: Pytoseiidae). A. swirskii*, like *Typhlodromus pyri (Acari: Pytoseiidae)*, is a type III generalist predator and feeds on several common pests such as spider mites (*Tetranychidae*), thrips, and whiteflies (McMurtry & Croft, 1997). *A. swirskii* was used due to difficulties in sourcing the *A. pyri* mites (high cost / low availability), ease of rearing *A. swirskii* in the laboratory, and common use in integrated pest management programs. It is also used in non-target toxicity testing in the literature (e.g. Döker & Kazak, 2019; Gadino et al., 2015; Lopez et al., 2015). These predatory mites can also survive on pollen, facilitating laboratory rearing and early-season establishment in crop systems (Lopez, 2023). For these reasons, *A. swirskii* is a highly effective biocontrol agent for integrated pest management programs in greenhouses and open fields globally (Calvo et al., 2015; Lopez, 2023). Pesticide treatments of crops may be toxic to *A. swirskii*, reducing its effectiveness in controlling pest populations (Fiedler & Sosnowska, 2014; Kim et al., 2018). Therefore, it is essential to understand how mixtures of pesticides used in the CSS may impact such beneficial predators. Effects of the 11 pesticide mixtures on juvenile mortality and reproduction were performed based on the standard IOBC protocol outlined for *Typhlodromus pyri* (Blümel et al., 2000).

7.2 Methodology

Treatments for the predatory mite testing were based on application rates (AR) in the CSS (**Table A2** in the **Annex**). The AR was determined based on preliminary data of the pesticide application patterns in each CSS. For each active substance, the AR was calculated as the geometric mean of the application rates used in the fields of the respective CSS. When data were unavailable for the particular active substance/CSS combination, the AR was derived from the recommended application rates of approved pesticides in the corresponding country.

The test evaluated the contact toxicity of the 11 pesticide mixtures on juvenile predatory mites and the subsequent effects on the reproduction of surviving adult females. Test arenas comprised 3 cm diameter bean leaf discs (*Phaseolus vulgaris*) treated with the pesticide mixture. Leaves were placed adaxial side down on cotton pads in the centre of a petri dish filled with water to provide moisture and prevent the escape of the mites. These arenas deviate from the standard protocol, which uses glass plates as test arenas (Blümel et al., 2000) but is similar to other studies testing the effects of pesticides on phytoseiid mites (e.g. Döker & Kazak, 2019; Gadino et al., 2015; Lopez et al., 2015).

Predatory mites were exposed to 6 treatments with 4 replicates each: A negative control (demineralized water), solvent control (4% acetone), positive control (dimethoate; 6 g a.s./ha), and the pesticide mixtures at AR/2, AR, and 2AR. All pesticide mixtures were

dissolved in acetone and diluted with demineralized water. Treatments were applied to leaf discs using a clean L-shaped cell spreader and allowed to dry for 30 minutes before the start of the experiment.

Two days before the experiment, *A. swirskii* eggs were collected from a stock colony initialized with mites purchased from a commercial supplier (Koppert B.V., Berkel en Rodenrijs, The Netherlands). Eggs were moved to a clean rearing unit, similar to those described by Overmeer (1985), and kept in a climate chamber at 25°C, 70% RH, and a 16:8 photoperiod. On day 0 of the experiment, protonymphs were collected from these rearing units and placed onto test arenas. Arenas were kept in the climate chamber for the duration of the experiment. On day 7, the number of dead mites, escaped mites (found dead either in the cotton pad or petri dish), and living males and females were counted. All eggs, larvae, and nymphs were removed from the arenas. Males were then transferred between replicates of the same treatment to achieve a sex ratio of at least 1 male:5 females in as many arenas as possible to ensure that all females would be mated. The number of eggs, larvae, and nymphs were counted on days 10, 12, and 14 to assess reproduction. Reproduction was calculated as the mean cumulative eggs per female using the formula proposed in the standard methods for *T. pyri* (Blümel et al., 2000). Both survival and reproduction were expressed relative to the solvent control to differentiate between the effect of the pesticide mixture and the effect of the solvent (acetone). A Kruskal-Wallis test was conducted to assess differences in survival and reproduction for each CSS. When significant effects were detected, a Dunnett post-hoc test was used for comparisons between treatments and CTs. Due to high mortality, the PT mixture was excluded from the reproduction analysis.

7.3 Results & Discussion

There was no significant difference in the mean survival between negative controls and solvent controls when considered together, suggesting that 4% acetone did not significantly impact the survival of *Amblyseius swirskii*. Additionally, the dimethoate treatment resulted in reduced survival compared to both negative and solvent controls, supporting the validity of the test. However, the toxicity of the dimethoate control was less than expected, as the same application rate should result in less than 50% survival in *Typhlodromus pyri*. This suggests that *A. swirskii* may be less sensitive to the compound (Blümel et al., 2000). No significant differences were observed in mean cumulative reproduction between day 7 and day 14 between the negative, solvent, and positive controls.

Pesticide toxicity data are limited for *Amblyseius swirskii* (Schmidt-Jeffris et al., 2021); therefore, the observed mixture toxicity could not be compared to single compounds. A significant difference between treatments and the solvent control was observed for the CH, ES, FR, NL, and PT mixtures (**Figure 7.1**). The PT mixture was especially toxic to *A. swirskii* juveniles, with a mean survival (relative to the control) of 7.69 ± 9.23% for the AR, and 15.4 ± 7.94 % for 2AR. Additionally, although no significant difference was detected, exposure to residues of the PT mixture at AR/2 resulted in survival of 20 ±

11.6%. Survival was also significantly lower than the solvent control at AR/2 for the FR and ES mixtures. The PT, FR, and ES mixtures contain sterol biosynthesis inhibiting fungicides (tebuconazole, difenoconazole/fenbuconazole, and difenoconazole, respectively) which have been found to have synergistic effects with insecticides in honeybees due to inhibition of cytochrome P450; however, such effects have not been explored in phytoseiid mites (Carnesecchi et al., 2019).

Reproduction was less sensitive to the pesticide treatments. Only the AR mixture at 2AR was significantly different from the solvent control. These results coincide with a meta-analysis that reported that for several phytoseiid mite species, pesticides have greater effects on juvenile mortality than on fecundity (Schmidt-Jeffris et al., 2021).

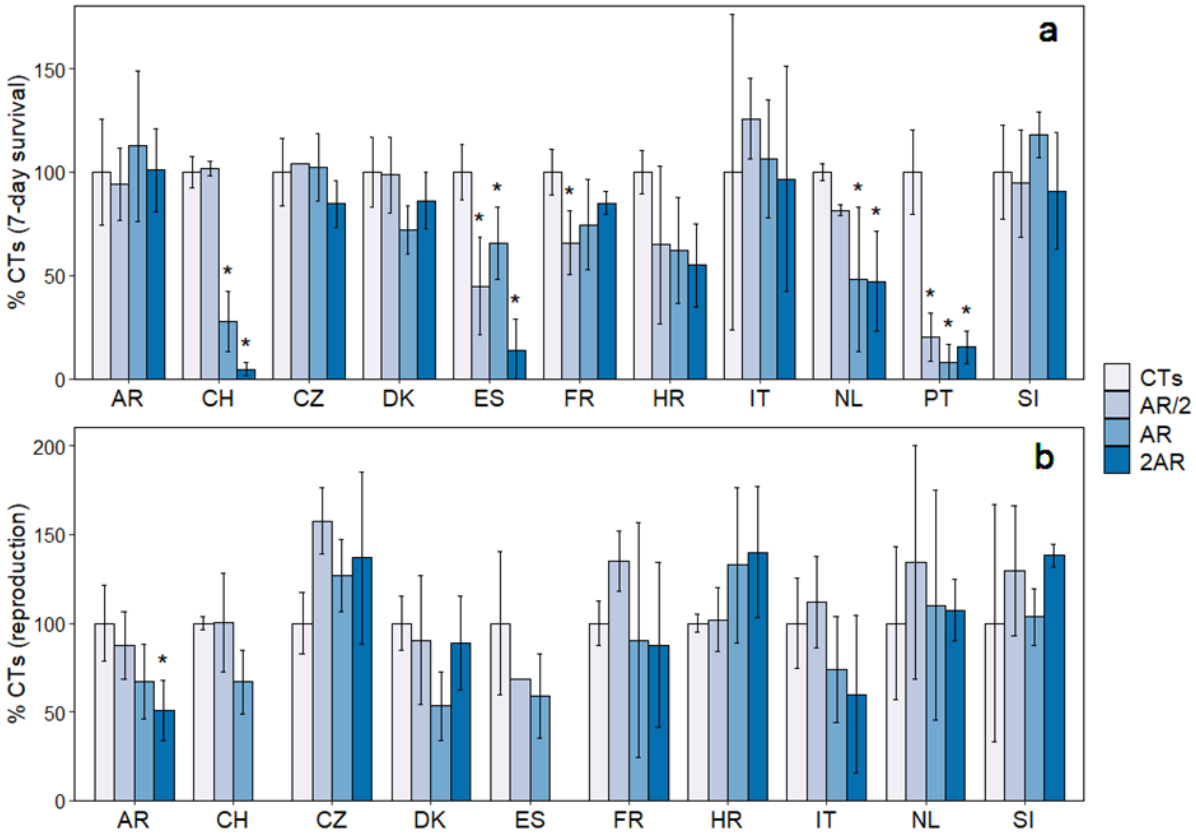

Figure 7.1: Effects of the 11 pesticide mixtures on a) mean 7-day juvenile survival, and b) mean cumulative reproduction per female, presented relative to the solvent control (mean ± sd). Asterisks indicate a significant difference from the solvent control (Dunnett's post-hoc, $p<.05$). For treatments: CTs: solvent control, AR: application rate. For the pesticide mixtures: AR: Argentina, CH: Switzerland, CZ: The Czech Republic, DK: Denmark, ES: Spain, FR: France, HR: Croatia, IT: Italy, NL: The Netherlands, and SI: Slovenia.

7.4 Conclusions

- Significant toxic effects on juvenile mortality were observed for the CH, DK, ES, FR, NL, and PT mixtures.
- *Amblyseius swirskii* juveniles were most sensitive to the PT mixture.

- Mortality at half of the application rate (AR/2) for the ES and FR mixtures suggests that the application of multiple compounds may impact the survival of *A. swirskii* in crops.
- Most mixtures did not impact the reproduction of surviving *A. swirskii* females, suggesting that reproduction is a less sensitive endpoint.
- There was a significant reduction in reproduction for *A. swirskii* exposed to the AR mixture at 2 times the application rate (2AR)

8 Terrestrial plants

8.1 Introduction

As primary producers, terrestrial plants are the base of the food web, producing energy for other trophic levels. Moreover, terrestrial plants are also responsible for providing habitat and shelter to other organisms and, in the case of leguminous plants, for hosting nitrogen-fixing bacteria. Pesticide exposure, especially herbicides, can cause deleterious effects on non-target terrestrial plants, and therefore, toxicity tests with plants are necessary. In tier I, the toxicity assessment of terrestrial plants involves the OECD 208 seedling emergence and seedling growth test (OECD, 2006a) and the OECD 227 vegetative vigour test (OECD, 2006b), as proposed by the Guidance Document on Terrestrial Ecotoxicology SANCO/10329/2002 (EC, 2002). Plant biomass, emergence, height of shoots, and visual observations are the endpoints analysed in these tests. Another standard test with the advantage of the short-term exposure (4 or 5 days depending on the species) is the ISO 11269-1 root growth inhibition test (ISO, 2012b). In this test, the root size of pregerminated seeds is measured as an indication of toxicity. The disadvantage of these tests is that they do not assess reproductive effects, such as seed production, flower formation, and fruit maturation (Arts et al., 2015). Nevertheless, they are very useful as a first screening of toxicity, when no information on the substance toxicity is available. Therefore, in the case of pesticide mixtures, short-term tests can be a useful approach for understanding the risks posed by the specific mixture being tested.

8.2 Methodology

Root growth inhibition test and **seedling emergence and growth** test were conducted based on the ISO 11269-1 (ISO, 2012b) and OECD 208 (OECD, 2006a) guidelines, respectively. One **monocotyledonous (*Triticum aestivum*)** and one **dicotyledonous species (*Lactuca sativa*)** were selected. For each mixture, 3 concentrations were used: MEC, PEC, and 5PEC. The negative control and the solvent control were tested, too. For the root growth inhibition test, 15 germinated seeds were sown in 200 g of soil. Plants were grown in a climate room for 5 days, and root size was measured. For the seedling emergence and growth test, 10 seeds were planted in 500 g of soil. Pots were placed in a climate room, and plants were checked daily for emergence. The tests were conducted for 14-21 days after 50% of control plants had emerged. At the end of this period, shoot was dried (60 °C, 24h) and weighted. A Kruskal-Wallis test was performed to compare the effects between the mixture concentrations and the controls, followed by a pairwise multiple comparison.

8.3 Results & Discussion

In the **root growth inhibition test**, wheat root length was significantly lower at MEC in ES, FR, SW, SI, mixtures, and at PEC and/or 5PEC in all CSS, except the HR mixture (**Figure 8.1**). Lettuce root length significantly decreased at PEC and 5PEC in ES, SI, CZ,

DK, AR, PT, and NL mixtures and at 5PEC in the IT mixture. The **root growth** seemed to be stimulated in the HR mixture for both plant species at MEC and PEC.

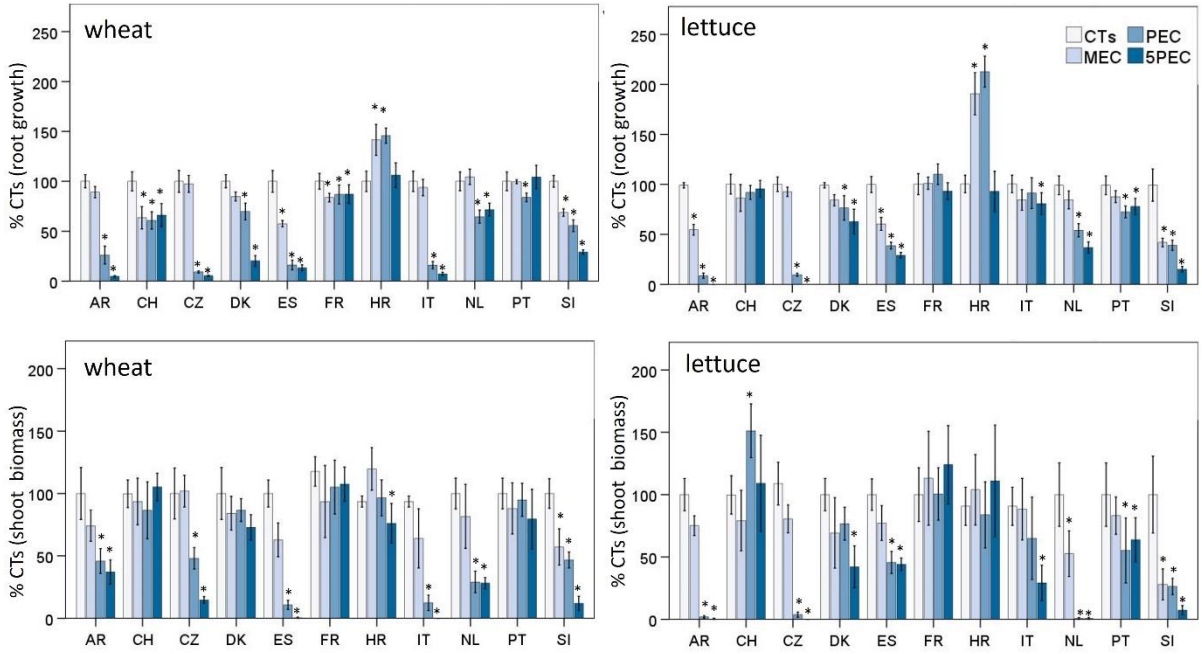

Figure 8.1: Root growth inhibition and shoot biomass (mean±SD) expressed as % solvent control in wheat (*Triticum aestivum*) and lettuce (*Lactuca sativa*). AR: Argentina; CH: Switzerland; CZ: Czech Republic; DK: Denmark; ES: Spain; FR: France; HR: Croatia; IT: Italy; NL: The Netherlands; PT: Portugal; SI: Slovenia.

In the **seedling emergence and growth** test, the effects on wheat seedling emergence were observed at PEC and 5PEC in the ES and IT mixtures and 5PEC in the SI mixture. The shoot biomass of wheat significantly decreased at MEC, PEC, and 5PEC in the mixture from SI, at PEC and 5PEC in mixtures from ES, IT, CZ, NL, and AR, and at 5PEC in the mixture from HR.

For lettuce, **seedling emergence** was affected in the CZ, NL, and AR mixtures at PEC and 5PEC. The shoot biomass of lettuce significantly decreased at MEC, PEC, and 5PEC in the mixtures from SI and NL, at PEC and 5PEC in the mixtures from ES, SI, CZ, PT, and AR, and at 5PEC in the mixture from IT.

The **root growth inhibition** in wheat was the most sensitive endpoint, for which significant effects were observed in 4 CSS at MEC and 7 CSS at PEC and 5PEC.

Pesticide effects on plants can be species-specific and endpoint-specific (Serra et al., 2019), which was in line with our results. For example, some mixtures (FR and CH) were toxic at MEC in the root growth test for both species. However, no toxicity was observed in the seedling emergence and growth test. Other mixtures (CZ and AR) showed toxicity to all endpoints tested at PEC. The mixture from SI was the most toxic, decreasing root growth and shoot biomass at MEC and affecting lettuce emergence at 5PEC.

8.4 Conclusions

- Overall, root growth was the most sensitive endpoint.
- Terrestrial plants were very sensitive to the exposure of pesticide mixtures in most CSS, with effects on seedling biomass and root growth at MEC.
- Effects occurring at MEC indicate that the presence of multiple pesticide residues in soil is an important route of exposure to be considered.

9 Bees

9.1 Introduction

Bees, including honeybees (*Apis mellifera*) and bumblebees (*Bombus terrestris*), are highly beneficial pollinators, supporting the cultivation of crops globally (Aizen & Harder, 2009; Potts et al., 2016). Observed bee declines have been attributed to several factors, including pesticide exposure (Goulson et al., 2015). Honeybees are used as an indicator of pesticide risk to other bee species in the approval of new compounds. The effects of single pesticides on honeybees have been widely studied (Benuszak et al., 2017; Cullen et al., 2019; Iwasaki & Hogendoorn, 2021). However, it is also important to consider the impacts of pesticide mixtures, as bees can be exposed to a combination of compounds when foraging for pollen and nectar (Sanchez-Bayo & Goka, 2016). The acute oral toxicity of the 11 pesticide mixtures was tested for honeybees following the OECD 213 acute oral toxicity test, with an endpoint of mortality 48 hours after exposure (OECD, 1998).

Both honeybees and bumblebees are eusocial, forming colonies consisting of a reproducing queen and genetically related female workers. The survival of these colonies relies on the reproductive capacity of the queen, as well as the combined efforts of workers in foraging for nectar and pollen and caring for the brood (Seeley, 2010). Sublethal concentrations of pesticides may negatively impact important aspects of honeybee colony function, such as foraging efficiency, which could, in turn, affect the survival of the colony (Gill et al., 2012; Prado et al., 2019). In particular, bumblebee colonies are reestablished every year by queens emerging from hibernation (Goulson, 2010). Therefore, queen production by bumblebee colonies, which can be reduced by insecticide exposure, may impact bumblebee populations in following years (Whitehorn, 2012). To assess the effects of chronic exposure to pesticides, honeybee and bumblebee colonies were given an adapted version of the NL pesticide mixture and evaluated for changes in the number of workers, brood, and stored food (honeybees), and changes in the amount and mass of workers and new queens (bumblebees). Honeybees were tested as EFSA indicators, while *Bombus terrestris* colonies were new SPRINT indicators (**Table 1.1**).

9.2 Methodology

9.2.1 Pesticide exposure

Concentrations used for honeybee and bumblebee experiments were based on the **predicted dietary exposure (PEQ)** of honeybees to the components of each pesticide mixture (**Table A2** in the **Annex**). The PEQ was calculated using a simplified equation for assessing dietary exposure proposed by the revised EFSA guidance for risk assessment of pesticides on bees: PEQ = AR/1000 × n × B, where AR is the application rate, n is the number of applications, and B is a constant that depends on application method (EFSA, 2023). B values were retrieved from the EFSA guidance for risk assessment in honeybees (EFSA, 2023). AR and n were calculated using preliminary data of application patterns in

the CSS. For each active substance, AR represented the geometric mean of the application rate in the CSS fields, and n represented the number of applications used in each field. When these data were unavailable, AR was derived from the recommended application rates of approved pesticides in the corresponding country, while n was derived from the maximum recommended applications. All active substances, except glyphosate, were dissolved in acetone and diluted with 50% (w/w) sucrose solution for a final acetone concentration of 3%. Glyphosate was directly dissolved in the sucrose solution used for the corresponding mixtures.

9.2.2 Acute effects on individual honeybees

The OECD 213 **acute oral toxicity** test was performed on honeybees, following the recommendation of the EFSA revised guidance on the risk assessment of plant protection products on bees (EFSA, 2023; OECD, 1998). Honeybees were fed 50% sucrose solution spiked with pesticides at 6 concentrations: PEQ/4, PEQ/2, PEQ, 2.5PEQ, 5PEQ, 10PEQ. Both negative (sucrose solution) and solvent controls (3% acetone) were included. To validate the method, Dimethoate was used as a positive control at a range covering the reported LD50 (0.10-0.35 µg/bee). Honeybees were collected from 3 healthy (by visual inspection) colonies (Inbuzz v.o.f.) and were placed in plastic boxes lined with filter paper, with 10 bees per box. Three replicates were included corresponding to the colony of origin, for a total of 30 bees per dose. The bees were moved to a dark climate chamber at 25 °C and 70% RH, given 50% sucrose solution, and acclimated to lab conditions for one day. At the beginning of the experiment, each box was given 100µl of treatment through a modified 1.5ml microcentrifuge tube. Four hours after the exposure, all treatments were replaced with 50% sucrose solution, which was provided ad libitum for the rest of the experiment. If the dose was not finished after 4 hours, the remaining amount was measured. Mortality and the number of bees exhibiting sublethal effects (paralysis, spasms, freezing) were recorded every 24 hours for 96 hours. The acute effects of the doses relative to the solvent control were analysed separately for each pesticide mixture using a Kruskal-Wallis test, as the data were not normally distributed. For CSS mixtures with significant effects, Dunnett's post-hoc was performed for comparisons with the solvent control.

9.2.3 Effects on honeybee colonies

Honeybee colonies were exposed to an adapted version of the NL pesticide mixture, including three insecticides: esfenvalerate, lambda-cyhalothrin, and pirimicarb. Ten honeybee colonies (Inbuzz v.o.f.) were kept at an apiary with ample natural resources near WU (51°57'17.8" N, 5°38'12.7" E). The colonies started in a 10-frame brood box (simplex measures), with similar amounts of bees, brood, and food, and each one healthy laying queen. Five colonies were exposed to 50% sucrose solution spiked with the pesticide mixture (0.5% acetone), and the other five were given a clean sucrose solution. Doses were 2.5PEQ for esfenvalerate and lambda-cyhalothrin; however, the dose of pirimicarb was reduced to PEQ/4 due to the availability of the compound (**Table A2**). The

corresponding concentration of sucrose solution given assumed that each colony contained 6,000 foragers consuming 10µl/bee/day (**Table 7.1**). All treatments were placed in an inverted glass jar on top of the colony, with holes in the lid for feeding.

Each colony was given 165 mL of (spiked) sucrose solution twice weekly for 6 weeks, from 4 August to 12 September 2023. Colonies were also given sugar paste (APIFONDA®) ad libitum. Colony development was measured using the Liebefeld method, a visual estimation of the area covered by honeybee workers, brood, and food using a 5 x 5 cm grid (Dainat et al., 2020). These measurements were performed 4 times: before exposure, mid-exposure, end of exposure, and 6 weeks post-exposure. The difference in number of workers, food cells, and brood cells between control and treated colonies was evaluated using a two-sample t-test and Wilcoxon rank-sum test when data were not normally distributed. The number of workers and wax comb cells in the colony were estimated assuming 1.24 bees/cm^2 and 4 cells/cm^2 (Delaplane et al., 2013).

Table 9.1: Concentration (mg/ml) of pesticides in sucrose solution given to honeybee (*Apis mellifera*) and bumblebee (*Bombus terrestris*) colonies, corresponding to the PEQ (µg/bee).

Species	Active substance	Treatment	Intended dose (µg bee^{-1} day^{-1})	Concentration (mg/ml)
Honeybees *Apis mellifera*	esfenvalerate	2.5PEQ	0.392	0.039
	lambda-cyhalothrin	2.5PEQ	0.233	0.023
	pirimicarb	PEQ/4	0.400	0.040
Bumblebees *Bombus terrestris*	esfenvalerate	2.5PEQ	0.392	0.010
	lambda-cyhalothrin	2.5PEQ	0.233	0.006
	pirimicarb	PEQ/4	0.400	0.010

The honeybees from the colony experiment were dissected and the characterization of their gut microbiota is ongoing. Then, the microbiota will be compared between control and treated colonies.

9.2.4 Effects on bumblebee colonies

Twenty standardized R&D bumblebee colonies with similar starting sizes (bees, brood, and food) and genetically similar queens (Koppert B.V.) were purchased and placed outdoors at the same apiary near WU used for the honeybees. These colonies were exposed to the same adapted NL pesticide mixture as the honeybee colonies twice weekly for 6 weeks (15 August – 22 September 2023). For bumblebees, the concentration of the treatment assumed that a colony of 100 bumblebees would receive 40uL/bee/day (**Table 9.1**). Colonies were given 10 mL of spiked or clean sugar solution at each feeding in plastic syringes with the tips removed. Bumblebee colonies were weighed once per week to estimate the size of the colony. Colonies were sacrificed following the final exposure, and the number and mass of workers and were recorded. Differences in the number and mass of worker bees, queens, and drones between control and treated colonies were assessed

with a Wilcoxon rank-sum test. A two-sample t-test was used to assess differences between control and treated colony masses at the end of the 6-week exposure period. Additive effects of each mixture were estimated with LD50 values from the literature, based on the concentration addition model used by Taenzler et al. (2023).

9.3 Results & Discussion

9.3.1 Acute effects on individual honeybees

Of the 11 mixtures tested, the CH, CZ, FR, and HR mixtures exhibited a significant toxic effect on *Apis mellifera*, and no significant effects were detected for the AR, DK, ES, FR, IT, PT, and SI mixtures (**Figure 9.1**). In particular, the CH mixture had the greatest effect, as survival was significantly lower than the survival of the solvent control for most doses, and nearly all bees were dead after 48 hours (**Figure 9.1**). This is as expected considering the toxicity of the single pesticides. The toxicity of this mixture is likely driven by the inclusion of emamectin benzoate, which has an acute LD50 of 0.002 µg/bee at 48 hours (Ulziibayar et al. 2022), which is several orders of magnitude less than the lowest dose tested (PEQ/4: 0.137µg/bee). Survival was significantly lower than the solvent control for the NL and HR mixtures at 2.5PEQ and above, which is as expected considering the LD50 of the components. For the HR mixture, all concentrations contained doses of phosmet higher than the LD50 (PEQ/4: 1.05µg/bee, LD50: 0.37µg/bee). In the NL mixture, the three insecticides were present at doses greater than the LD50, starting at 2.5PEQ (esfenvalerate), 5PEQ (pirimicarb), and 10PEQ (lambda-cyhalothrin).

The CZ mixture significantly reduced survival for the highest dose (10PEQ), which is greater than expected based on the toxicity of the components. The CZ mixture contains two insecticides (lambda-cyhalothrin, acetamiprid, flupyradifurone) and two fungicides (tebuconazole and spiroxamine). Both tebuconazole and spiroxamine are sterol biosynthesis inhibitors. Such fungicides have been found to have synergistic effects with insecticides in honeybees, due to inhibition of cytochrome P450 monooxygenases (Carnesecchi et al., 2019; Taenzler et al., 2023). Tebuconazole was also present in the HR mixture, but high doses of phosmet masked any potential synergism.

PEQ was calculated as a function of application rate, using an equation retrieved from the new EFSA guidelines for risk assessment in bees (EFSA, 2023). However, this equation is simplified and recommended for screening steps of exposure assessment, as it does not include factors such as food consumption, sugar content of nectar, and landscape characteristics. As such, the calculated PEQ is conservative, and therefore may overestimate field conditions. Regardless, as the lowest doses of the CH mixture (PEQ/4 and PEQ/2) resulted in low mean survival, the toxicity of this mixture may merit further investigation.

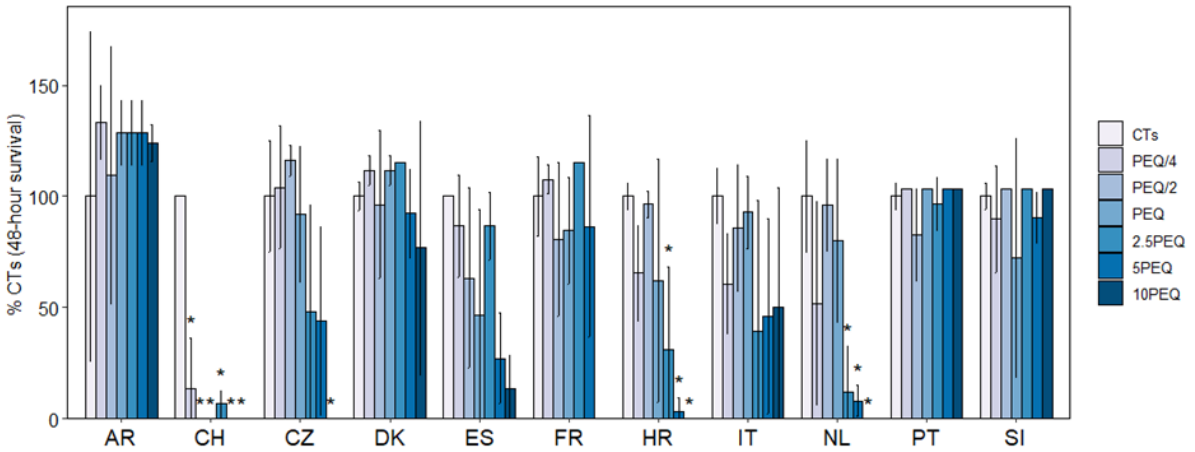

Figure 9.1: Effects of pesticide mixtures on survival of *Apis mellifera* 48 hours after oral exposure. Bars represent the mean survival expressed as a percentage of the survival of the solvent control, and error bars represent the standard deviation. Asterisks indicate a significant difference in survival with the solvent control (Dunnett's post-hoc, p<.05). CTs: solvent control, AR: Argentina, CH: Switzerland, CZ: Czech Republic, DK: Denmark, ES: Spain, FR: France, HR: Croatia, IT: Italy, NL: The Netherlands, PT: Portugal, SI: Slovenia.

9.3.2 Effects on honeybee colonies

Before exposure, the mean estimated number of worker bees, honey cells pollen cells, capped brood cells, open brood cells, and egg cells did not differ between control and treated colonies, suggesting that all colonies started with similar conditions. Three weeks into the 6-week exposure period, treated colonies had fewer worker bees than control colonies (**Figure 9.2**).

There was a 47.6% reduction in the mean estimated number of worker bees counted before exposure. The toxicity of this mixture may be driven by esfenvalerate, of which the intended dose was above the recorded oral acute LD50 (0.21 µg/bee). The mean number of worker bees in each colony before exposure was greater than the estimated 6,000 (9,331±1,323). Therefore, the starting dose was likely lower than intended, potentially ranging from 0.17-0.22 µg/bee. However, these estimated doses are still in the range of the LD50 of esfenvalerate for honeybees. After three weeks, treated colonies also had significantly fewer capped brood cells than control colonies, while there were no significant differences in egg, open brood, honey, and pollen cells (**Figure 9.2**). This suggests that at the middle of the exposure period, the mixture did not significantly affect egg production by the queen and development to open brood. The significant difference in capped brood could result from fewer worker bees available to attend to the brood. After the 6-week exposure, treated colonies had significantly fewer worker bees, capped brood, open brood, eggs, and honey, indicating the continued negative effect of the pesticide mixtures on honeybee survival, the reduced ability of the colony to store honey for the winter, and a reduction in brood production relative to the controls. The effects of lambda-cyhalothrin have been found to negatively affect the learning, memory, and homing ability of honeybees, which could affect the foraging ability of bees and reduce food storage (Liao et al., 2018). However, there was no significant difference in honey cells per worker bee

between control and treated colonies, suggesting that the reduction in food storage is linked to the decrease in the colony's population.

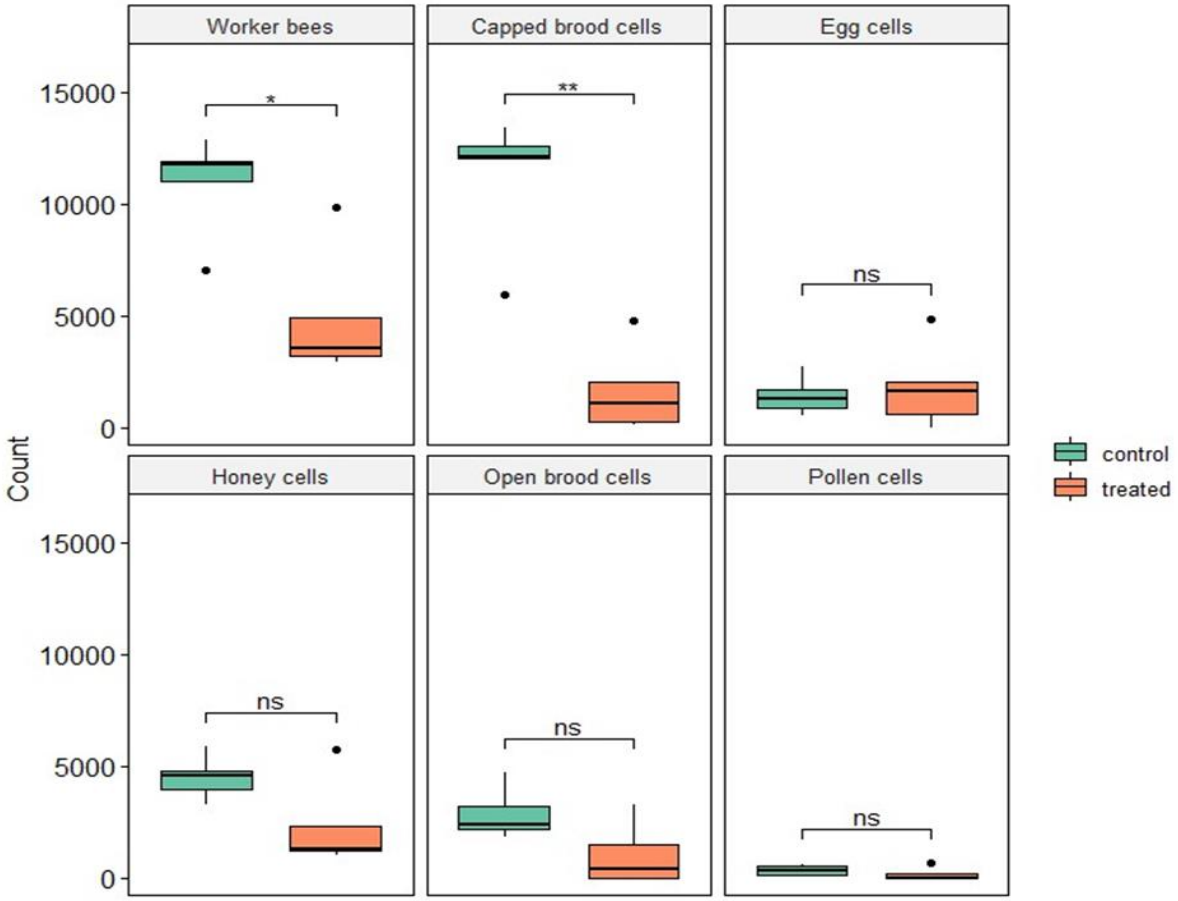

Figure 9.2: Estimated number of worker bees and cells containing stored food (honey and pollen) and different stages of brood (eggs, open, and capped) counted in honeybee (*Apis mellifera)* colonies 3 weeks into the 6-week exposure to a mixture of esfenvalerate, lambda-cyhalothrin, and pirimicarb. Asterisks indicate the p values resulting from either a t-test or Wilcoxon rank-sum test. ns: $p > .05$, *: $p \leq .05$, **: $p \leq .01$, ***: p .001.

9.3.3 Effects on bumblebee colonies

The NL mixture had little to no toxic effect on *Bombus terrestris* colonies over the 6 week period. Colony weight was not significantly different between control and treated colonies at the end of the 6-week treatment (**Figure 9.3**). Additionally, both the mean number and mean mass of workers, queens, and drones did not significantly differ between control and treated colonies, indicating the similar conditions of *Bombus terrestris* in both groups (**Figure 9.3**). Some toxicity of the NL insecticide mixture was expected, as the concentration of lambda-cyhalothrin was 0.006 mg/ml, while significant effects on queen and worker mortality have been observed in greenhouse studies at 0.003 mg/ml (Cueppens et al., 2015). However, the intended doses of esfenvalerate and pirimicarb (**Table 9.1**) were an order of magnitude less than the acute oral LD50 for *B. terrestris* (5.52 and 8.5µg/bee, respectively; Cabezas & Farinós, 2022; Gretenkord & Drescher, 1993).

The lack of observed effects of the pesticide mixture may be due to low treatment consumption. Bumblebee colonies were kept in an outdoor apiary, meaning that nectar foraged by the colony may have diluted the consumption of pesticide treatment. However, bumblebee colonies consumed significantly less of the provided sucrose solution at each feeding than the control colonies. A repellent effect of both esfenvalerate (Cabezas & Farinós, 2022) and lambda-cyhalothrin (Thompson & Wilkins, 2003) has been observed in bees, suggesting that these compounds may explain the reduced feeding of bumblebees.

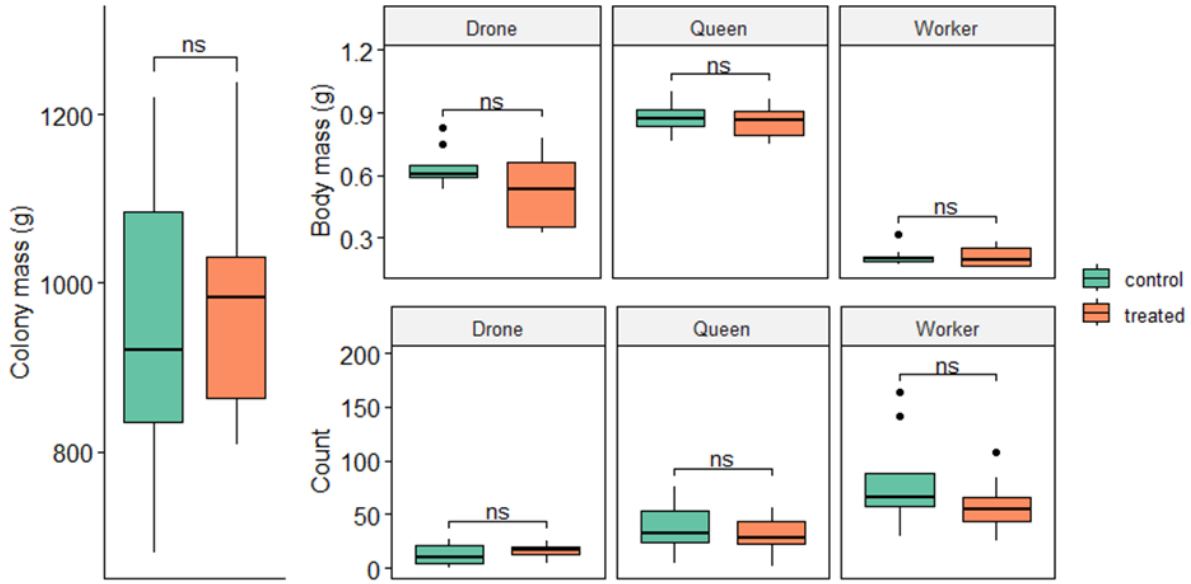

Figure 9.3: Differences in colony mass (left), mean body mass (top right), and number of bees (bottom right) between bumblebee (*Bombus terrestris*) colonies exposed to a mixture of esfenvalerate, lambda-cyhalothrin, and pirimicarb for 6 weeks. Worker bees, queens, and drones (males) were counted and weighed separately. Brackets indicate significance resulting from either a Wilcoxon rank-sum test (number and mass of bees), or a two-sample t-test (colony mass).

9.4 Conclusions

- Most doses of the CH mixture resulted in significantly low survival of individual honeybees, potentially due to the high toxicity of emamectin benzoate.
- Toxicity of the NL, HR, and CZ mixtures at higher doses may also be due to high concentrations of certain insecticides.
- Toxicity of the CZ mixture was greater than expected at 10PEQ, suggesting potential synergism of compounds.
- The NL mixture of insecticides was highly toxic to honeybee colonies, possibly driven by the presence of esfenvalerate at the LD50.
- No significant effects of the NL mixture were observed in bumblebee colonies, but low consumption of treatment suggest a repellent effect.

10 Novel soil microbial indicators

10.1 Introduction

Pesticides are still used in agriculture ecosystems to increase yields or ensure plant health. While they reach their designated target organism, soils are suspect to contamination by residues or application errors. There, soil microorganisms provide and maintain soil functions such as nutrient cycling and litter degradation (Hobara et al., 2014; Madsen, 2011). Many studies found that microorganisms can be influenced by pesticides (Karpouzas et al., 2016; Meidl et al., 2024). But in today's risk assessment there are only two standardized methods for soil microorganisms (OECD 216: nitrogen transformation test, OECD 217: carbon transformation test). To address this shortcoming, new methods for soil microorganisms in pesticide risk assessment were tested and evaluated in microcosm experiments in the SPRINT project.

10.2 Methodology

In different Tier 1 experiments (description see below), new ecotoxicological indicators for terrestrial ecosystems were tested, verified and optimized. Soils which were used for Tier 1 experiments in Hohenheim had no previous pesticide history (soils came from a field close to Hohenheim or from the LUFA) and sieved to 2 mm. To allow the soil microorganism to acclimate, soil was pre-incubated in the dark at room temperature for one week. In all experimental set ups a treatment without pesticides was used as control for microbial abundance and activity. Pesticides were chosen because of our knowledge from previous studies (Wirsching et al., 2020; Wirsching et al., 2022; Wirsching et al. 2023; Ditterich et al., 2013), their use in the case study sites from SPRINT or their occurrence in agricultural soils. Pesticides were applied at different concentration as single or multiple stressors.

Pesticide residues were extracted following Wimmer et al. (2022) or following an adaption of the QuEChERS method as described by Silva et al. (2019). According to DIN EN ISO 16072:2011-09 soil microbial respiration was measured via titration. Soil enzyme activities based on MUF substrates were used to quantify the potential of soil microorganisms to degrade different organic substances in soils (modified ISO 20130 (ISO, 2018) following Marx et al. (2001)). As indicators for bacterial and fungal abundances phospholipid fatty acids were used as described by Mäder et al. (Revision resubmitted, August 2024) following a modified ISO/TS 29843 (ISO, 2010) according to Frostegård et al. (1991). In addition, DNA was extracted using the FastDNA spin Kit for soil (MP Biomedicals) following the company's protocol. Quantitative real-time PCR was used as molecular method to quantify specific pesticide degraders like glyphosate degraders and MCPA degraders (Wirsching et al., 2022; González-Valenzuela & Dussán 2018; Bælum et al., 2008) and functional genes involved in C-, N- and P-cycling (N – fixation: *nifH* (Rösch et al., 2002), nitrification of archaea/ bacteria: *amoA* AOA / AOB (Leininger et al., 2006; Rotthauwe et al., 1997), denitrification: *nirK* / *nirS* (Henry et al., 2004; Throbäck et al., 2004), acid phosphatase: *phoN* (Bergkemper et al., 2016)).

In a Tier 1 the experiment (A) described in **Chapter 4**, ten different pesticide mixture (according to the case study sites) in three concentrations (MEC, PEC, 5PEC) with four replicates per treatment were used and soil samples for day 28 and 56 were analyzed. The soil was collected from Unifarm (Wageningen University, the Netherlands), air-dried, and sieved at 5 mm. The soil characteristics were: pH_{CaCl2} of 6.2±0.1, soil organic carbon of 0.6%, 1% clay, 8% silt, and 89% sand. The enzyme activities were measured based on MUF substrates and molecular methods were used as new approach to quantify functional genes involved in nutrient cycles.

According to OECD 2000 a Tier 1 microcosm experiment (B) was established comparing the OECD 216 test with current available functional gene assays, to test the molecular properties as potential new methods for environmental risk assessment. As pesticide, the persistent fungicide boscalid at five distinct concentrations (1x, 2x, 5x, 10x PEC_{Acc}) with four replicates per treatment (Stache et al., in prep.) was used. Destructive soil sampling was performed on days 0, 3, 7, 14, 21, 28, 56.

To test a multiple pesticide approach, a Tier 1 microcosm experiment (C) was set up (according to OECD 216) with the herbicide glyphosate, the fungicides azoxystrobin and tebuconazole and the insecticide cypermethrin, in concentrations of 1x PEC and 10x PEC (according to wheat cultivation). Additionally, a mixture treatment of all pesticides with the 1x PEC concentration was applied. Destructive soil sampling was performed on days 0, 7,14, 28 and 56. The analyses are currently running.

In addition, a joint Tier 1 experiment (D) between UH and the University of Palermo was conducted to test the functional gene assay in a multi-stressor approach, which involves citric waste water from lemon orchards, copper and the insecticide abamectin. Laboratory work and data acquisition are still in progress.

In a further Tier 1 microcosm approach (E) the effects of two additional pesticides, herbicide MCPA and fungicide difenoconazole, next to glyphosate on the soil microbial community as well as the degradation of ^{13}C labelled glyphosate were investigate (Mäder et al., Revision resubmitted, August 2024).

To follow the effects of pesticides on special functions of soil microorganisms, an additional Tier 1 microcosm experiment (F) was conducted. Since the reaction of soils to multiple pesticides can depend on the quality of the organic matter, an experiment was set up with two different litter qualities (fresh and aged maize litter). Pesticides used were the herbicide prosulfocarb and the fungicide boscalid as well as the mixture of the two. Next to measurements of the pesticide residues, potential enzyme activity, the microbial biomass, microbial activity and community structure, the substrate use was followed utilizing the natural ^{13}C label of the maize leaves.

To verify the applicability of the MUF method and the quantification of the functional genes as soil microbiological indicators for testing pesticide toxicity, UH will measure the enzyme activities based on the MUF substrates and use the molecular methods to quantify the functional genes in a Tier 2 microcosm experiment (G) designed and described by WU

(Dennis Knuth). These measurements will be carried out in cooperation between UH and WR over the next few months (until the end of 2024) in UH.

10.3 Results & Discussion

All the results showed that it is necessary to set up the right control treatments (minimum soil with solvent and as additional control only soil) in order to guarantee stable control values as reference values for ecotoxicological tests. The important outcome of these experiments was that the current recommended duration of the OECD test may be insufficient to detect potential ecotoxicological effects (**Figure 10.1**). In the early phase of incubation (until 28 days), possible toxic effects can be masked by the addition of the readily available organic C source. Furthermore, metabolites of the pesticide may have a greater impact than the parent active substance (Vasileiadis et al., 2018).

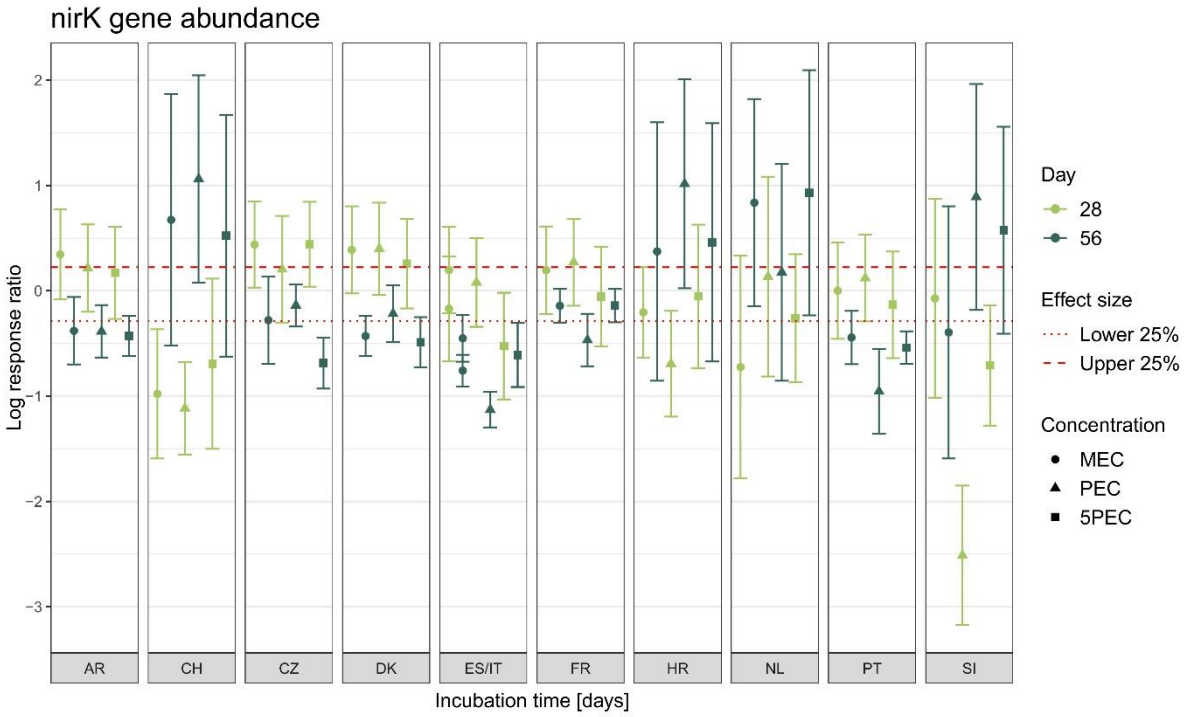

Figure 10.1: Copy numbers of the functional gene nirK (per g dry matter) targeting the nitrite reductase shown as response ratio whereas zero corresponds to the control soils. Standard deviations are given as error bars; dotted redlines show the lower and higher 25% effects; Mixtures for Argentina (AR), Switzerland (CH), Czech Republic (CZ), Denmark (DK), Spain and Italy (ES/IT), France (FR), Croatia (HR), The Netherlands (NL), Portugal (PT) and Slovenia (SI).

In addition, the results showed a pesticide-induced reduction in *phoN* copy numbers (**Figure 10.2**), whereas the OECD test did not reveal any effects. These results showed that the functional gene assay seems to be a more sufficient method to detect boscalid toxicity against non-target soil microorganisms than the standardized OECD 216 test. Additionally, the microbial response to the pesticide was monitored via CO_2 measurements over the experimental period (**Figure 10.3**) and only showed significant differences to the control in the late state of the experiment. This leads us to the view that ecotoxicological tests require a longer duration (at least 56 days).

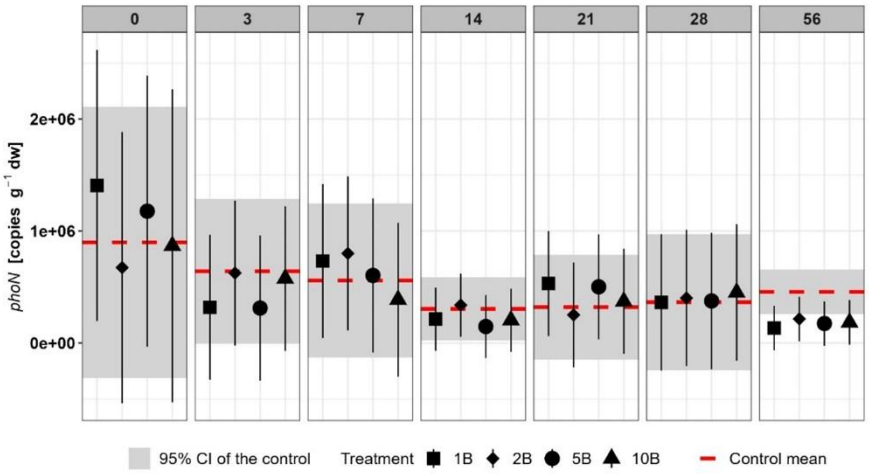

Figure 10.2: Abundance of acid phosphatase gene sequence (phoN) over the experimental period (days 1 to 56). Symbols indicate estimated marginal mean values and error bars 95% confidence interval. The red dotted line represents the estimated marginal mean of the control and the grey rectangle the 95% confidence interval. B stands for the fungicide boscalid, and the numbers represent the 1x, 2x, 5x and 10x concentration of the PECAcc (predicted environmental concentration accumulated for one year) (experiment B).

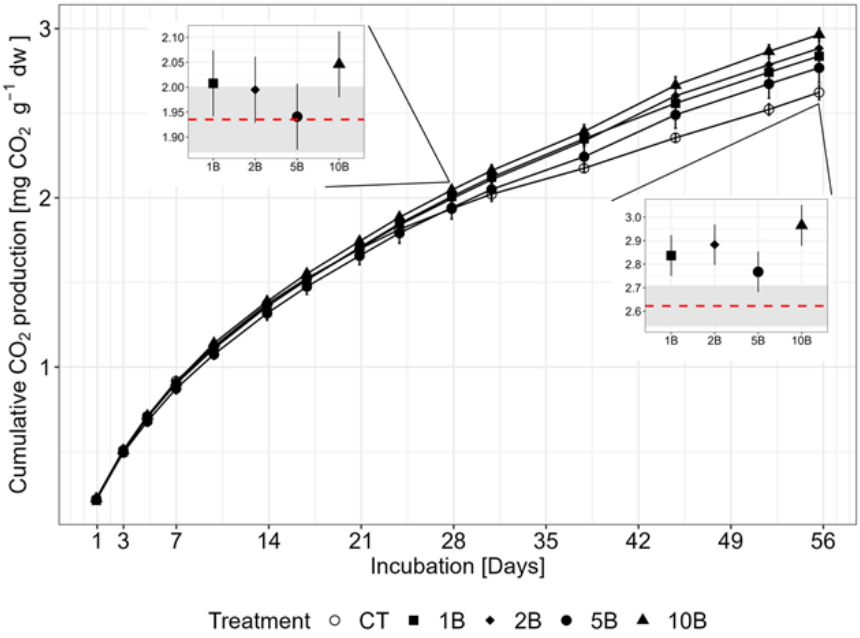

Figure 10.3: Cumulative CO_2 production over the experimental period (days 1 to 56). Symbols indicate mean values and error bars the standard deviations. The two smaller pictures show the outcome of the Dunnett's test; symbols indicating the estimated marginal mean and the error bars the 95% confidence interval of the pesticide treatments; the red dotted line represents the estimated marginal mean and the grey rectangle the 95% confidence interval of the control. B stands for the fungicide boscalid, and the numbers represent the 1x, 2x, 5x and 10x concentration of the PECAcc (predicted environmental concentration accumulated for one year) (experiment B).

Further results of a Tier 1 experiment (E) highlight, that interactions between multiple pesticides in soils can lead to both additive and antagonistic effects on microbial parameters, where not only the number of pesticides but also the effects of the individual components influenced the microbial community. This could be seen in the PLFA data as

well as in the abundance of the functional genes involved in the degradation of specific pesticides.

The results of the Tier 1 experiment F (**Figure 10.4**) indicated that the quality of maize litter modulates the pesticide-microorganism interaction. The combination of the two pesticides had both additive and antagonistic effects as well as expressing single pesticide patterns. This highlights the complexity of the effects pesticide mixtures under environmental conditions.

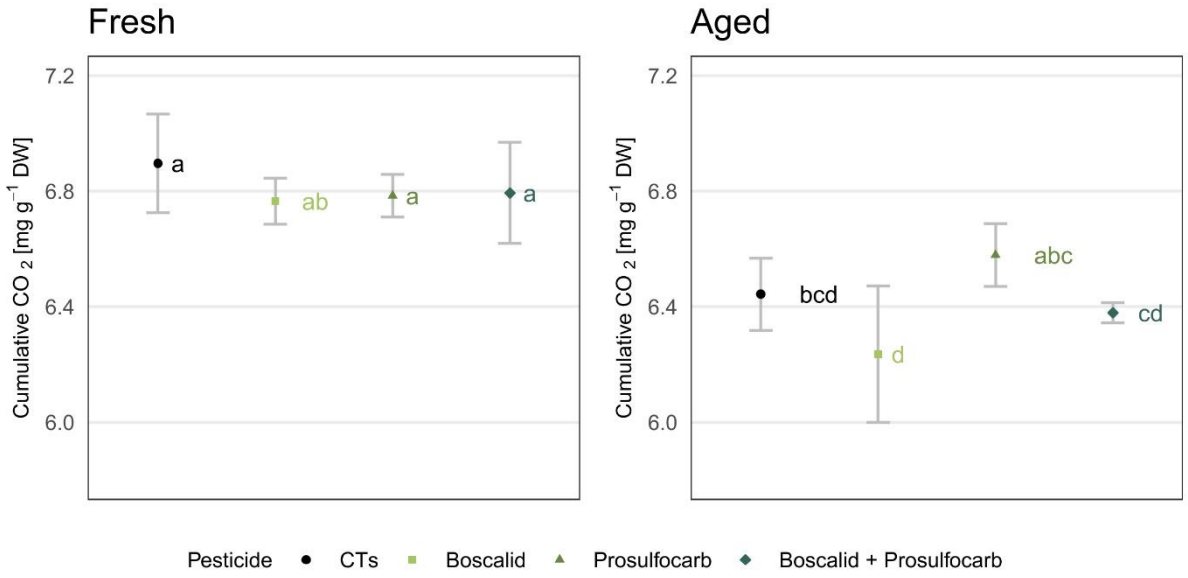

Figure 10.4: Cumulative CO_2 production on day 56 of the experiment for unaltered as well as altered maize treatments. CTs (no pesticides added), boscalid (2 mg kg^{-1}), prosulfocarb (20 mg kg^{-1}) and the mixture with the same concentrations were used as pesticide treatments. Symbols indicate mean values and error bars the standard deviations. Different letters indicate a significant difference (experiment F).

10.4 Conclusions

- New indicators for soil microorganisms are promising, but the final recommendation for future usage is still in progress.
- The duration of standardized experiments should be extended since possible toxic effects can be masked by adding easily available substrate.

11 Soil microcosms

11.1 Introduction

Microcosms are test units designed to increase the complexity and realism of experiments, in comparison to single-species assays (van den Brink et al., 2005). In microcosms, different trophic levels are employed to simulate, within its limitations, natural ecosystems. In this way, microcosm experiments can assess the toxicological effects of substances considering the interactions between species and account for indirect effects. For example, earthworms change the chemical and physical structures of soils, which in turn can be beneficial to plants and microorganisms. Moreover, the presence of several species and different soil processes can alter the environmental fate of the substances, such as pesticides (Burrows & Edwards, 2004).

In the current European legislation, it is stipulated that high-tier assessments must be conducted for pesticides and PPP presenting an unacceptable risk at tier 1 (EC, 2013a; EC, 2013b). High-tier assessments (e.g., semi-filed and field studies) closely resemble natural conditions and environment, including the species' interactions and mode of exposure (Edwards, 2002), and therefore are the most realistic approach. Nevertheless, high-tier studies may have low reproducibility, high variability, and are extremely costly (Burrows & Edwards, 2002). For those reasons, multi-species microcosm has been proposed as an intermediate tier, between tier I (single-species) and high-tier experiments, for the risk assessment of pesticides (Ernst et al., 2024).

11.2 Methodology

Using a tiered approach, the effects of the most toxic CSS on earthworms were assessed in a microcosm experiment. The results from reproduction tests with the different earthworm species showed that the CZ mixture was one of the most toxic, and therefore it was chosen for the microcosm experiment. The test was conducted using the native earthworm species *Lumbricus rubellus*. The design was based on previous work conducted at MUNI (Šudoma et al., 2019). Five concentrations were used, namely CT, CTs, MEC, PEC, and 5PEC. A natural soil collected at Unifarm (Wageningen, The Netherlands) was used in the experiments. The soil was pre incubated for 4 days (50% WHC, in dark, at room temperature). Ten adult earthworms and 8 pre-germinated lettuce seeds were added to each microcosm pot, containing app. 1 kg of moist soil. After 14, 28, and 56 days, 4 pots per concentration were sacrificed for the analysis. The lettuce plants were removed from the soil, washed, and dried with towel paper. The dry weight of leaves was recorded as plant biomass. The adult earthworms were collected, washed, dried with a towel paper, and weighed (fresh weight). On the 56d, the number of juveniles and cocoons was counted. The carbon and nitrogen mineralization were measured as described in chapter 8. The growth rate in earthworms was calculated as the difference between final and initial weights divided by initial weight, and it was analysed by a two-way ANOVA, using concentration and exposure time as independent variables, followed by Tukey's post hoc

test. The number of cocoons was analysed by one-way ANOVA, followed by Tukey's post hoc test. The number of juveniles was analysed by a Kruskal-Wallis test, followed by a pairwise multiple comparison.

11.3 Results & Discussion

No effects on lettuce germination and biomass were observed, with seedling emergence ranging from 6 to 8 per pot. In earthworms, survival was affected only at 5PEC, where 90% and 100% mortality were observed after 28 and 56 days, respectively. The growth rate and number of cocoons significantly decreased at PEC and 5PEC, while the juvenile number decreased with increasing concentration; however, it was not significant (**Figure 11.1**).

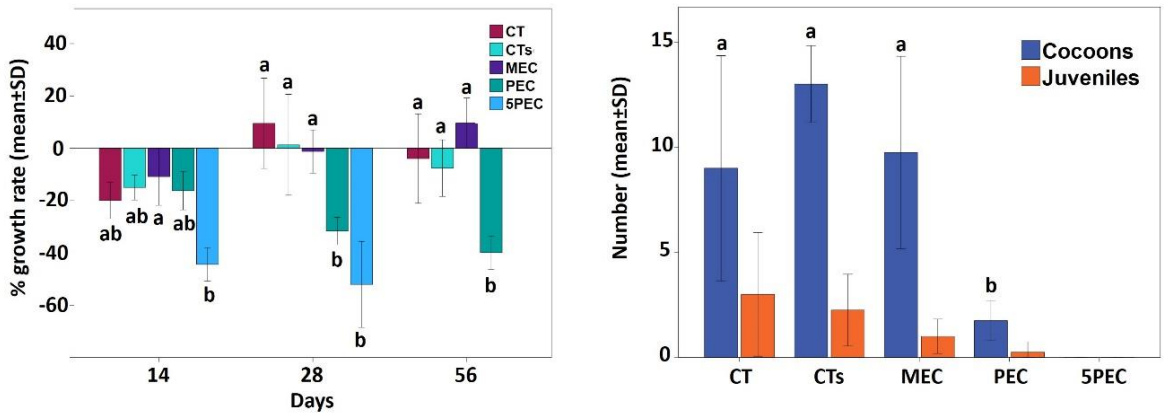

Figure 11.1: Growth rate (%) of earthworms (*Lumbricus rubellus*) after 14, 28, and 56 days of exposure in negative control (CT), solvent controls (CTs), measured environmental concentration (MEC), predicted environmental concentration (PEC), and 5 times PEC (5PEC) based on the results of residues in CZ. Letters indicate significant differences (Tukey's post hoc test, $p<0.05$). Bars indicate standard deviation.

The results of the microbial activity in the microcosms can be seen in Figure 6. No effect on basal respiration (BR) was observed after 14 and 28 days. However, after 56 days, the BR increased in all concentrations, with deviation from solvent control of 36%, 91%, and 145% at MEC, PEC, and 5PEC, respectively. A similar trend was observed for the substrate-induced respiration (SIR). The deviation from control was >25% only after 56 days, with values of 48% and 81% at PEC and 5PEC, respectively. The potential ammonification (PAMO) could not be measured after 14 days, as ammonium levels did not increase with the addition of L-arginine. No effects on PAMO were observed at MEC and PEC; however, at 5PEC, the deviation from solvent control was 62% and 27% after 28 and 56 days, respectively. The potential ammonia oxidation (PAO) showed a deviation from the control of 55% and 90% at PEC and 5PEC after 28 days. However, it decreased to <25% after 56 days.

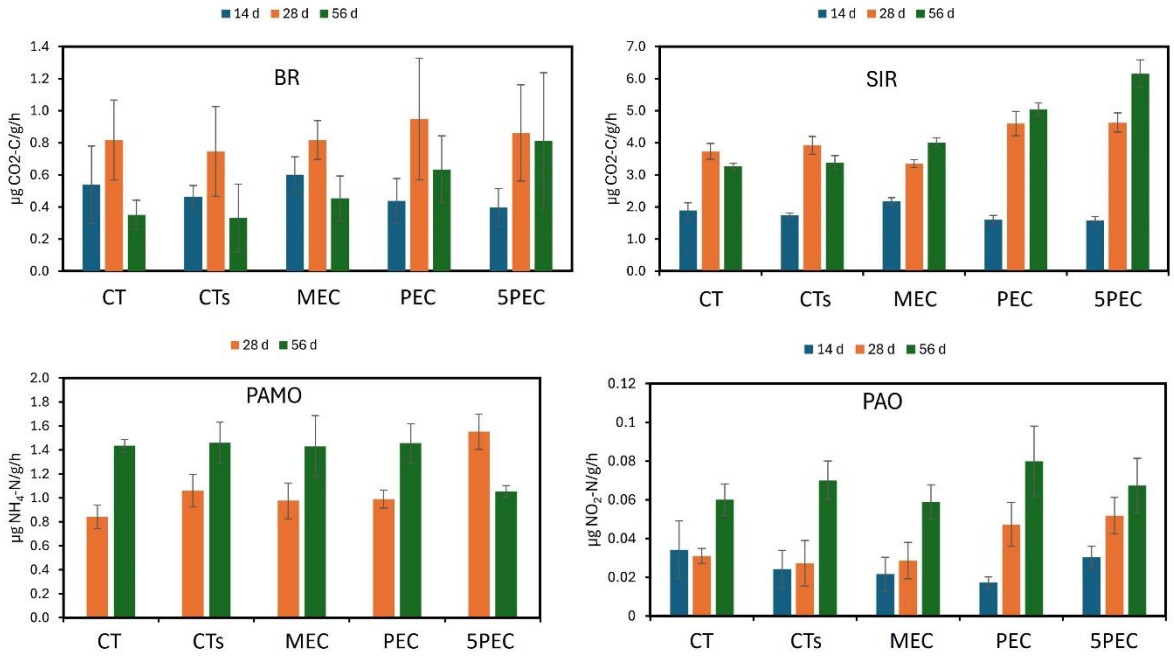

Figure 11.2: Effects on microbial activity in the microcosms after 14, 28, and 56 days. Mean values of basal respiration (BR), substrate induced respiration (SIR), potential ammonification (PAMO), and potential ammonia oxidation (PAO) are given and bars represent the standard deviation. CT: negative control; CTs: solvent control; MEC: measured environmental concentration; PEC: predicted environmental concentration; 5PEC: five times PEC.

11.4 Conclusions

- Effects on earthworm growth and reproduction were observed at PEC exposure. The toxicity of the pesticide mixture on survival and growth increased in comparison with the single species test with *L. rubellus* (**Chapter 4**).
- For microbial activity, the basal respiration was the most sensitive, with effects observed at MEC, while for the other endpoints, the microbial activity was affected at PEC and 5PEC exposures.
- The effects on carbon mineralization were only evident after 56 days. For nitrogen mineralization, the effects peaked after 28 days, while after 56 days, no effect on PAO was observed and effects on PAMO decreased but still were > 25% deviation from control.

12 Chemical analyses of soil concentrations in the tests

Determining the pesticides in soil samples from the ecotoxicity tests with earthworms, springtails, and microcosms were conducted. Soil samples were frozen at the beginning of the experiments (i.e., immediately after spiking) and compared to the nominal concentrations.

All soil samples were thawed and homogenized. The sample was split into two parts: 2g for the determination of glyphosate AMPA and the remaining 5-10 g for the screening of multi-residues.

Glyphosate and AMPA were determined using the modified method described Yang et al. (2015), Goscinny et al. (2012), and Kaczyński & Łozowicka (2015) using LC-MS/MS (Agilent 1200 chromatographic system coupled to a ESI/QqQ Agilent Triple Quad 6410 mass spectrometer (Agilent, Santa Clara, CA,USA)). Samples were spiked with isotope-labelled standard glyphosate and AMPA solution and extracted with 10 mL of 0.6 M KOH. The samples were shaken for 50 minutes and then centrifuged (5 min, 3500 rpm). The derivatisation step: 0.5 mL of the supernatant, 40 µL of 6 M HCl, 0.25 mL of 5% borate buffer and 0.25 mL of FMOC-Cl were added to a plastic tube. The tubes were shaken and incubated for 30 min at room temperature. The reaction was stopped by adding 25 µL of concentrated formic acid. All samples were transferred to plastic LC vials integrated with 0.45-µm PFTE filters. The limit of quantification (LOQ) was 0.05 mg/kg dry soil.

The other pesticide residues were extracted using an adaptation of the QuEChERS approach to the samples, as described by Silva et al. (2019) and analysed by LC-MS/MS and GC-MS/MS (MS-MS XEVO TQ-S (Waters) coupled to a 7890 GC from Agilent Technologies). Samples were spiked with D6-metolachlor (used as surrogate standard) and mixed with 5 mL Millipore water and 10 mL acetonitrile. The plastic tube with this mixture was agitated for 1 min, after which QuEChERS salts (4 g magnesium sulfate, 1 g sodium chloride, 1 g sodium citrate, 0.5 g disodium citrate) were added to the tube. The tube was then vortexed and centrifuged (5 min, 3500 rpm) and the supernatant was collected. Part to be analyzed using LC-MS/MS and part to be analyzed using GC-MS/MS. For the LC-MS/MS analysis the samples were diluted with water and added directly into a LC filter vial to be analyzed. For the GC-MS/MS analysis, there was an extra clean-up step with dispersive sorbents. The cleaned supernatant was transferred into a conical glass vial to be analyzed. The LOQ ranged from 0.004 to 0.05 mg/kg dry soil for LC-MS/MS, and from 0.001 to 0.01 mg/kg dry soil for GC-MS/MS.

In the earthworm tests, the initial concentration of 88 soil samples from the test with *E. fetida* were measured. For each concentration, two replicates were measured, and one replicate for each control (negative and acetone). In **Figure 12.1**, the average recovery (% of nominal concentration) versus the nominal concentration is displayed. Recoveries in most samples (85.8%) were below 80% of nominal concentration, while recoveries in 8.4% of samples were between 80 to 120%, and recoveries in 5.8% of samples were above 120%.

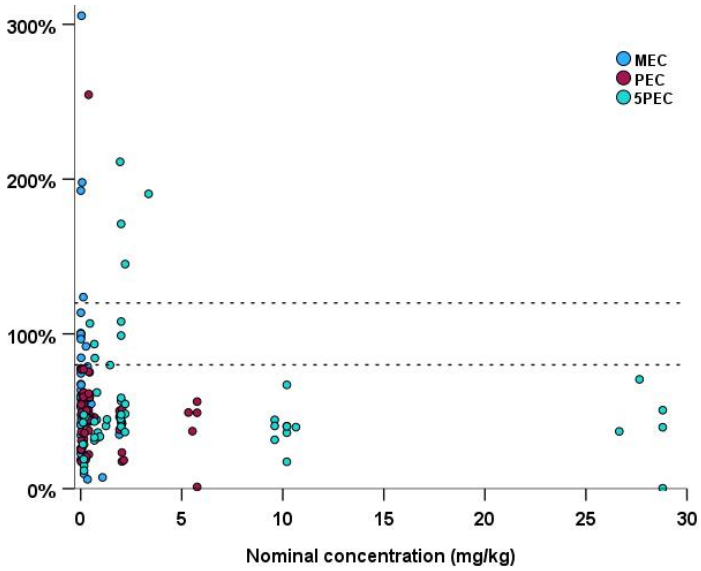

Figure 12.1: Recovery of the pesticides in samples (n=88) from 11 CSS in the test with earthworms and their nominal concentration in the median environmental concentration (MEC), predicted environmental concentration (PEC), and 5 times PEC (5PEC).

In the springtail test, the initial concentration of pesticides was measured in 90 soil samples and the final concentrations were measured in 75 samples. The samples were analyzed in triplicates for each concentration. The samples from 6 CSS have been measured and the samples from the other 5 CSS are being processed. The recovery of initial concentration versus nominal concentration is displayed in **Figure 12.2**. The recoveries in 48.8% of the samples were below 80% of nominal concentration, while 37.5% of samples had recoveries ranging from 80 to 120% and 13.8% of samples had recoveries above 120%.

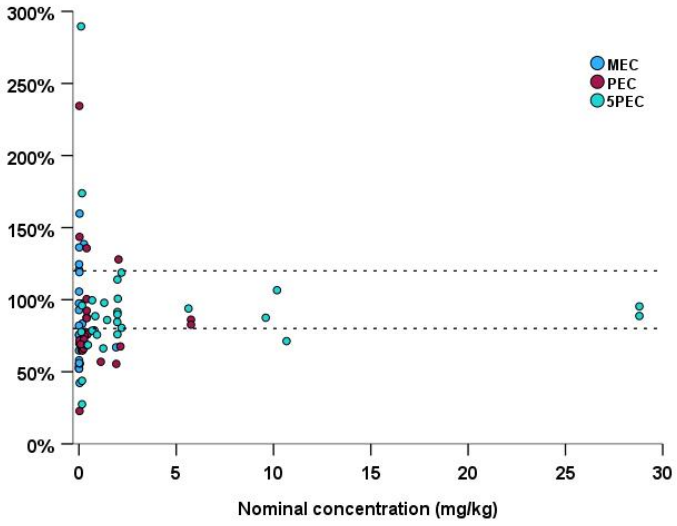

Figure 12.2: Average recovery of the pesticides in samples (n=90) from 6 CSS in the test with springtails and their nominal concentration in the median environmental concentration (MEC), predicted environmental concentration (PEC), and 5 times PEC (5PEC).

The initial and final concentrations were measured at the beginning of the experiment and after 28 days (**Figure 12.3**). For most pesticides, a low degradation occurred, with similar initial and final concentrations. Only acetamiprid, lambda-cyhalothrin, and phosmet showed faster degradation. Acetamiprid and phosmet have DT50 of 12 and 9 days, respectively. Lambda-cyhalothrin, on the other hand, has a high DT50 (174 days), and therefore it is difficult to determine what caused the fast degradation.

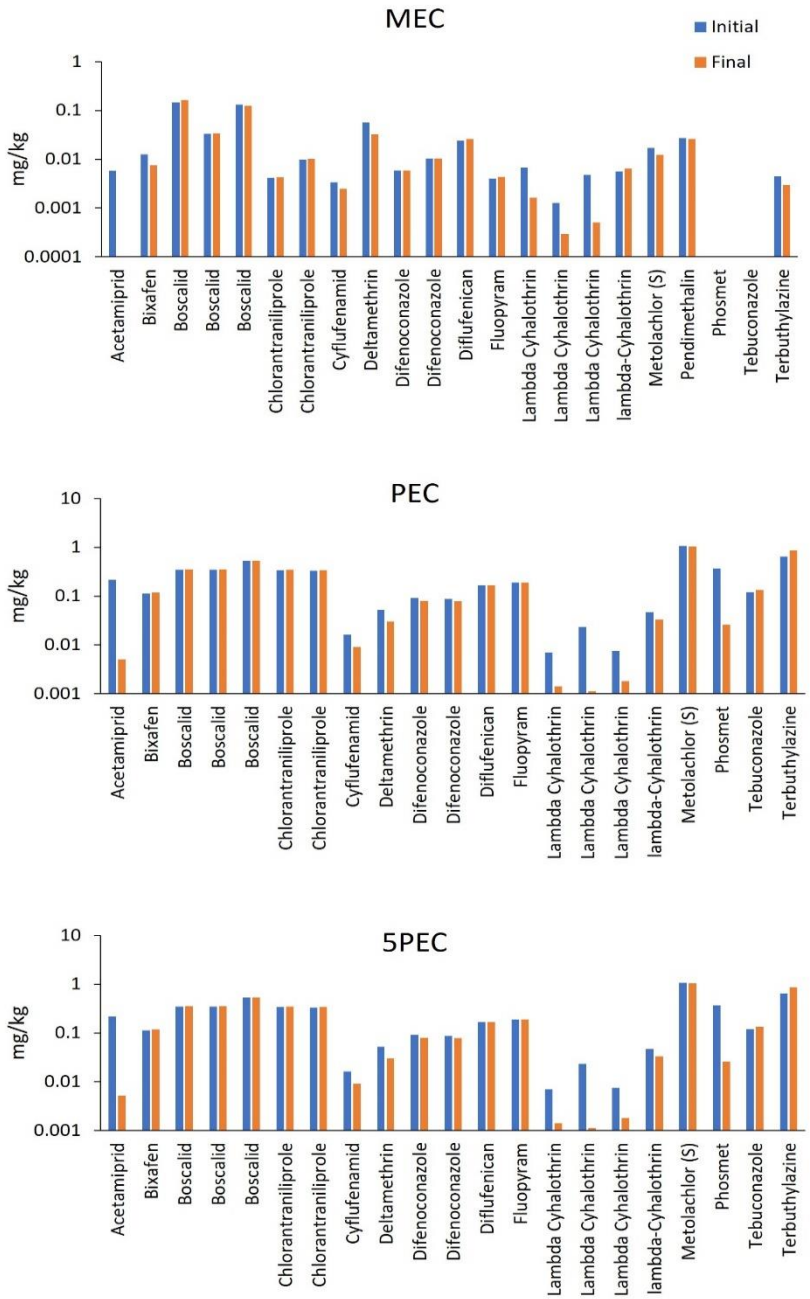

Figure 12.3: Initial and final concentrations of the pesticides from the test with springtails in the median environmental concentration (MEC), predicted environmental concentration (PEC), and 5 times PEC (5PEC) in 6 CSS. Empty bars indicate that the values were below the limit of quantification (LOQ).

In the microcosm test, the initial concentration of the pesticides was measured in a total of 15 samples. For each concentration, 3 replicates were measured. The recoveries were below 80% of the nominal concentration in 60% of the samples (**Figure 12.4**). In 33.3% of samples, the recoveries ranged from 80 to 120% of nominal concentration, while 6.7% of samples had recoveries above 120% of the nominal ones.

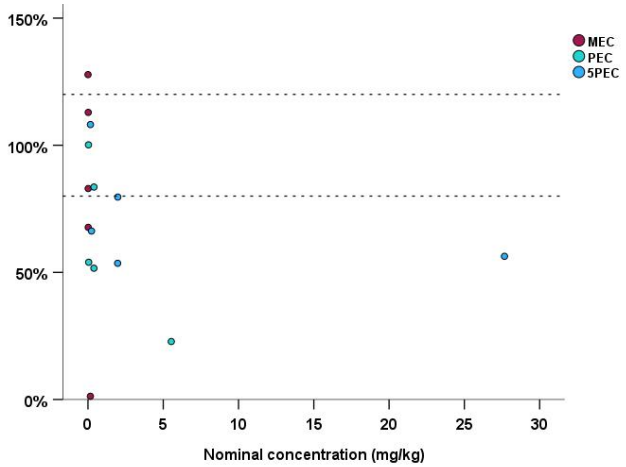

Figure 12.4: Average recovery of the pesticides in the samples from the microcosm experiments and their nominal concentration in the median environmental concentration (MEC), predicted environmental concentration (PEC), and 5 times PEC (5PEC).

13 Conclusions

13.1 Summary of the findings

Significant effects (i.e., > 25% deviation from the control) on **soil microbial activity**, assessed as carbon and nitrogen mineralization (**Chapter 4**), were observed in **6 CSS**. The **carbon mineralization** was affected in 5 mixtures (**CZ, ES/IT, FR, PT**) after 28 days of incubation, but only at 5PEC. These mixtures had in common the **fungicides metrafenone, tebuconazole**, and/or **boscalid**. After 56 days, only one mixture (**PT**) still caused adverse effects, which was no further observed after 84 days. The **nitrogen mineralization** was affected in only 2 mixtures (**CZ, NL**) at **PEC**, and in the case of **CZ**, the effects were observed until 84 days of incubation. The similarity between CZ and NL was the presence of **boscalid** and **strobilurin fungicides**. It can be concluded that nitrogen mineralization was more sensitive than carbon mineralization. However, 4 mixtures affected only carbon mineralization. No effect was observed after 84 days, which is within the time frame of 100 days for recovery of microbial activity in the risk assessment of microorganisms.

In the reproduction test with **springtails** (**Chapter 5**), no toxicity was observed in 6 CSS (AR, CH, CZ, DK, NL, SI). These outcomes were expected since the reproduction NOEC of the 5 pesticides in each mixture, reported by EFSA, were lower than their highest concentrations in soil (5PEC). Pesticide mixture from **5 CSS** caused effects on springtails. The effects on reproduction were observed at **PEC** and 5PEC in the **ES/IT, FR, and PT** mixtures (having the insecticide **chlorantraniliprole**, highly toxic to springtails, and the fungicides **boscalid and difenoconazole**), and at 5PEC in the **HR** mixture. These mixtures had at least one pesticide with NOEC values exceeding the exposure concentrations (PEC or 5PEC). These results align with the EU Commission's assessment of mixtures, which found that normally, a few pesticides in mixtures are reported to drive most of the toxicity (EC, 2020). Moreover, the effects occurred at **environmentally relevant concentrations** (i.e., the worst-case scenario PEC), which may be alarming, considering that no toxicity should be expected based on a single exposure to those pesticides. Although no toxicity was observed in MEC treatment, indicating that the median concentrations of pesticide real residues in soils were likely to cause no adverse effects on springtails, these results must be carefully interpreted. According to values observed in WP2 and published by Knuth et al. (2024), 35% of the measured concentrations exceeded the PEC values, with the concentration in the soil samples being up to 10 times higher than PEC.

For the **earthworms**, the reproduction tests were conducted with the standard *E. fetida* and native *L. rubellus*, and cast production and burrow activity were tested in glass mesocosms with *L. terrestris* (**Chapter 6**). The results showed that the anecic species was less sensitive than the standard and native epigeic species. However, the sensitivity of the two epigeic species was comparable and varied depending on the mixture. For the standard species, effects were observed only at 5PEC, while for the native species,

reproduction was affected at **MEC** (**NL**) and **PEC** (**CZ, DK**). The tests with *L. terrestris* also showed that survival, growth, cast production, and burrow activity significantly decreased at **PEC** (**CZ**). In general, observing **significant effects at MEC and PEC levels is alarming** because these concentrations are assumed safe from the EFSA-based assessments.

For beneficial insects testing, the contact toxicity test conducted on the predatory mite *Amblyseius swirskii* (**Chapter 7**) indicated that survival was significantly affected at or above the application rate (AR) for the **CH, DK, NL, and PT** mixtures. For the **ES and FR** mixtures, a significant reduction in survival was observed at **AR/2**. This is **alarming**, considering the AR is considered safe based on EFSA-based assessments. Reproduction appeared to be less sensitive to the pesticide mixtures, with a significant reduction in cumulative eggs/female observed for the **AR** mixture at 2AR. However, the **PT** mixture, which was highly toxic to juvenile mites, was excluded from the reproduction analysis due to the lack of surviving females.

In the test with **terrestrial plants** (**Chapter 8**), all mixtures were found to be toxic when considering all endpoints and species. The least toxic mixture (HR) caused effects only at one endpoint at 5PEC. Two mixtures (**CH, FR**) caused only effects on wheat root growth at **MEC**, while the other **8 mixtures caused several effects** on different species and endpoints at **MEC or PEC**. In general, observing **significant effects at MEC and PEC levels is alarming** because these concentrations are assumed safe from the EFSA-based assessments. The pesticide mixtures used in the terrestrial plant tests were composed of mostly **herbicides**. Due to the high number of herbicides with different characteristics (systemic, broad-spectrum) and mode of action (photosynthesis inhibitors, growth inhibitors, amino acid inhibitors, cell division inhibitors), it is difficult to pinpoint the components in the mixture most responsible for the effects observed or if synergetic effects could have occurred. Moreover, some pesticides (e.g., fungicides) in the mixture could have caused **indirect effects on plants**. These results highlight the risk posed by multiple pesticide residues in agricultural soil to non-target terrestrial plants.

For the **mortality tests on individual honeybees** in **Chapter 9**, significant acute effects of the pesticide mixtures were observed for the **CH, CZ, FR, and HR** mixtures after 48 hours. The CH mixture was the most toxic, as survival was affected at **PEQ/4**, while the HR and FR mixtures had a significant effect at 2.5PEQ. However, all three mixtures contained at least one compound at or above the LD50. Although the **CZ** mixture was only toxic at 10PEQ, the effect on survival was greater than expected. This, combined with the presence of **insecticides and sterol biosynthesis inhibiting fungicides** known to have synergistic effects, suggests possible interaction between components. High mortality was observed in **honeybee colonies** after 6 weeks of exposure to **esfenvalerate, lambda-cyhalothrin, and pirimicarb**, which may have been due to the presence of esfenvalerate at approximately its LD50. The reduction in stored honey was likely due to the decrease in worker bees rather than other sublethal effects. No differences were observed between **bumblebee colonies** control and treated colonies but treated colonies consumed significantly less sucrose solution, suggesting a repellent effect of the **insecticides**.

For the **novel microbial indicators** in **Chapter 10**, the effects of pesticide mixtures were observed in the several tests performed. The abundance of **functional gene nirK** exceeded the critical value (>±25% deviation control) in **all mixtures** considering both timepoints (28 and 56 days) and concentrations (**MEC, PEC, and 5PEC**). Moreover, the mixture of **boscalid and prosulfocarb** changed the CO_2 production when compared to their single exposures in soils amended with aged maize litter. Overall, the experiments with functional genes showed that effects were more prominent after extended duration (i.e., 56 days).

The **microcosm test** (**Chapter 11**) was conducted with the most toxic mixture in the earthworm reproduction tests (**Chapter 6**). Besides earthworm survival, growth, and reproduction, lettuce emergence and biomass and microbial activity were also assessed in the microcosm. Similarly to the results reported in Chapter 6, the mixture from **CZ** was highly toxic to the **native earthworm *Lumbricus rubellus***. After only 28 days of exposure, earthworm growth was significantly affected at **PEC**, and survival was significantly affected at 5PEC. The reproduction decreased after 56 days at **PEC** and 5PEC as well. Moreover, effects on **microbial activity** were observed only after 28 days for nitrogen mineralization and only after 56 days for carbon mineralization. Delayed effects on microbial activity are not currently considered in the standardized tests and risk assessment.

13.2 Comparisons across the tests

The tests with earthworms (**Chapter 6**) and springtails (**Chapter 5**), where the same mixtures and concentrations were applied, showed that most mixtures were toxic to only one of the two groups. Out of the 8 CSS found to be toxic to earthworms, only PT mixture was toxic to springtails as well. This can be explained by the fact earthworms are most sensitive to fungicides and springtails to insecticides. In both groups, 'more than additive effects' was suggested. In earthworms, the mixture from SI showed higher toxicity than expected based on NOEC values of the single pesticides, while for springtails that was observed in the mixtures from PT and FR.

The native earthworm species *L. rubellus* was exposed to CZ mixture in the reproduction test (**Chapter 6**) and microcosm test (**Chapter 11**). The results showed that the toxicity of CZ mixture to *L. rubellus* was higher in the microcosm test than in the reproduction test. These results highlight the importance of the multi-species assay. This test also provided further information on soil processes, as shown by the effects on carbon and nitrogen mineralization.

The same mixtures were also applied in the tests assessing the microbial activity (**Chapter 4**) and novel soil microbial indicators (**Chapter 10**). Higher sensitivity was observed when the novel microbial indicators were used as endpoints, with effects observed at MEC, while the nitrogen and carbon mineralization were affected at PEC and 5PEC, respectively.

13.3 Limitations of the results

In WP4, a whole-mixture approach was followed, with some modifications to adapt to the study of the 11 CSS, where priority pesticides were chosen in each CSS. Although the whole-mixture approach is an appropriate choice for assessing complex mixtures, **information is lost if the mixture composition changes with time**. Therefore, the pesticide selection followed a risk-based approach to include in the final mixtures the 5 pesticides posing the most risk to different ecological groups. This allowed us to study real and relatively complex mixtures of pesticides while keeping the number of test units to a minimum.

One major limitation of this approach, however, is the **lack of information on the contribution of each pesticide to the toxic effects observed**. Therefore, we compared the results obtained with the data on single pesticide exposure, as reported by EFSA and the literature. In this case, **different exposure scenarios**, such as soils with different properties, should be considered, as they can influence the toxicity outcomes. Moreover, **data availability might be a problem**. For many pesticides, toxicity data is unavailable for all the species, and sometimes, **only formulations** have been tested for a specific endpoint.

13.4 Outlook

The use of **alternative (native) species** and the testing in the multi-species systems was found to be extremely important when assessing the effects of pesticide mixtures. For example, 3 mixtures were toxic to the native earthworm species while presenting no toxic effects to the standard *E. fetida* (**Chapter 6**).

Higher-tier testing was shown as more sensitive compared to single species tests. In the microcosm experiment, a higher risk was suggested for the organisms (earthworm *L. rubellus* and microbial activity) compared to the single species tests (**Chapters 4, 6, 11**).

The results of microbial activity (**Chapter 6**) and novel microbial indicators (**Chapter 10**) suggested that **prolonged exposure** should be recommended. The effects of the mixtures were often not observed after 28 days, when, according to the OECD protocols, the tests are ended. Therefore, any effects observed only after 28 days would have been missed.

Finally, the **mixtures where synergetic or more than additive effects** were suggested should be **further explored**. That includes the toxic effects of **SI mixture to earthworms, FR and PT to springtails, and CZ to honeybees**.

Last but not least, it should be carefully explored how it is possible that significant effects at MEC and PEC levels were observed in several cases of our testing. These concentrations are assumed safe from the EFSA-based assessments. The effectiveness of the established EFSA approaches to protect the terrestrial ecosystems against harmful impacts of pesticide mixtures should be re-checked.

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Annex

Pesticide mixtures for the terrestrial ecotoxicological assessment

Table A1: Pesticide mixture selection per CSS for soil invertebrates.

CSS	Pesticides	mg/kg MEC	PEC	5PEC	MEC (IT)
ES	Oxyfluorfen	0.14	1.92	9.60	0.05
IT	Chlorantraniliprole	0.03	0.44	2.19	0.01
	Difenoconazole	0.02	0.14	0.68	0.01
	Lambda-cyhalothrin	0.02	0.03	0.16	0.01
	Boscalid	0.02	0.40	1.98	0.02
PT	Chlorantraniliprole	0.01	0.44	2.19	
	Boscalid	0.18	0.40	1.98	
	Glyphosate	0.79	5.76	28.8	
	Difenoconazole	0.005	0.14	0.68	
	AMPA	1.91	2.04	10.1	
FR	Chlorantraniliprole	0.01	0.44	2.19	
	Boscalid	0.03	0.40	1.98	
	Difenoconazole	0.01	0.14	0.68	
	Cyflufenamid	0.004	0.02	0.12	
	Glyphosate	0.26	5.76	28.8	
CH	Difenoconazole	0.01	0.14	0.68	
	Methoxyfenozide	0.01	0.09	0.46	
	Myclobutanil	0.01	0.67	3.36	
	AMPA	0.34	2.04	10.1	
	Pirimicarb	0.02	0.16	0.80	
HR	Boscalid	0.13	0.40	1.98	
	Phosmet	0.01	0.40	2.00	
	Acetamiprid	0.01	0.29	1.45	
	AMPA	0.52	2.04	10.1	
	Deltamethrin	0.04	0.02	0.11	
SI	Bixafen	0.01	0.17	0.84	
	S-Metolachlor	0.04	1.92	9.60	
	AMPA	0.07	2.04	10.18	
	Tebuconazole	0.01	0.19	0.93	
	Terbuthylazine	0.01	1.13	5.63	
CZ	Boscalid	0.02	0.40	1.98	
	Thiophanate-methyl	0.16	5.53	27.6	
	Lambda-cyhalothrin	0.002	0.03	0.16	
	Dimoxystrobin	0.02	0.05	0.24	
	Azoxystrobin	0.01	0.39	1.97	
NL	Lambda-cyhalothrin	0.00	0.03	0.16	
	Boscalid	0.02	0.40	1.98	
	Azoxystrobin	0.02	0.39	1.97	
	Bixafen	0.02	0.17	0.84	
	Prosulfocarb	0.06	5.33	26.6	
DK	Boscalid	0.01	0.40	1.98	
	AMPA	0.12	2.04	10.1	
	Diflufenican	0.02	0.25	1.25	
	Fluopyram	0.01	0.26	1.30	
	Pendimethalin	0.05	2.13	10.6	
AR	Glyphosate	0.54	5.76	28.8	
	Lambda-cyhalothrin	0.01	0.03	0.16	
	AMPA	1.33	2.04	10.1	
	Azoxystrobin	0.01	0.39	1.97	
	Methoxyfenozide	0.01	0.09	0.46	

Table A2: Pesticide mixture selection per CSS for non-target arthropods and bees.

CSS		Predatory mites (g a.i./ha)			Bees (µg a.i./bee)					
	Pesticides	**AR/2**	**AR**	**2AR**	**PEQ/4**	**PEQ/2**	**PEQ**	**2.5PEQ**	**5PEQ**	**10PEQ**
ES	Lambda-cyhalothrin	21.2	42.5	84.9	0.11	0.23	0.46	1.14	2.29	4.57
	Acetamiprid	50.0	100	200	0.48	0.96	1.92	4.80	9.60	19.20
	Metalaxyl-M	116	232	464	0.47	0.94	1.87	4.68	9.36	18.71
	Difenoconazole	39.5	79.1	158	0.25	0.51	1.01	2.53	5.06	10.12
	S-Metolachlor	480	960	1920	1.54	3.07	6.14	15.36	30.72	61.44
PT	Fluxapyroxad	22.5	45.0	90.0	0.10	0.20	0.41	1.01	2.03	4.05
	Glyphosate	360	720	1440.0	1.62	3.24	6.48	16.20	32.40	64.80
	Spirotetramat	37.5	75.0	150.0	0.17	0.34	0.68	1.69	3.38	6.75
	Tebuconazole	43.1	86.2	172.3	0.30	0.59	1.19	2.97	5.95	11.90
FR	Lambda-cyhalothrin	6.2	12.4	24.8	0.03	0.06	0.11	0.28	0.56	1.12
	Difenoconazole	14.5	29.1	58.2	0.11	0.23	0.46	1.14	2.28	4.56
	Fenbuconazole	18.8	37.5	75.0	0.08	0.17	0.34	0.84	1.69	3.38
	Trifloxystrobin	26.1	52.2	104.5	0.13	0.25	0.51	1.27	2.54	5.07
	Glyphosate	245.8	491.6	983.3	1.11	2.21	4.42	11.06	22.12	44.25
CH	Emamectin benzoate	15.2	30.4	60.8	0.14	0.27	0.55	1.37	2.74	5.47
	Difenoconazole	42.7	85.3	170.7	0.33	0.66	1.32	3.30	6.61	13.22
	Myclobutanil	41.2	82.4	164.8	0.66	1.32	2.64	6.61	13.22	26.43
	Pirimicarb	120	240	480	1.08	2.16	4.32	10.80	21.60	43.20
	Tebuconazole	61.3	122.6	245.2	0.72	1.45	2.89	7.23	14.46	28.92
IT	Lambda-cyhalothrin	11.6	23.1	46.3	0.04	0.07	0.15	0.37	0.74	1.48
	Pendimethalin	455	910	1820	1.46	2.91	5.82	14.56	29.12	58.24
	Propamocarb	530	1060	2120	1.70	3.39	6.78	16.96	33.92	67.84
	Bixafen	38.5	76.9	153.8	0.12	0.25	0.49	1.23	2.46	4.92
HR	Phosmet	205.0	409.9	819.9	1.04	2.07	4.14	10.35	20.71	41.41
	Acetamiprid	12.5	25.0	50.0	0.06	0.11	0.23	0.56	1.13	2.25
	Trifloxystrobin	30.3	60.6	121.2	0.19	0.39	0.77	1.93	3.86	7.72
	Glyphosate	7.5	15.0	30.0	0.07	0.14	0.27	0.68	1.35	2.70
	Tebuconazole	62.5	125.0	250.0	0.28	0.56	1.13	2.81	5.63	11.25
SI	S-Metolachlor	607.6	1215.1	2430.3	1.94	3.89	7.78	19.44	38.88	77.77
	Thiencarbazone-methyl	13.0	25.9	51.9	0.04	0.08	0.17	0.42	0.83	1.66
	Bixafen	37.9	75.7	151.5	0.20	0.41	0.82	2.04	4.08	8.15
	Tebuconazole	62.5	125.0	250.0	0.40	0.80	1.60	4.00	8.00	16.00
CZ	Lambda-cyhalothrin	3.8	7.5	15.0	0.01	0.02	0.05	0.12	0.24	0.48
	Tebuconazole	82.6	165.2	330.4	0.26	0.53	1.06	2.64	5.29	10.57
	Acetamiprid	33.5	66.9	133.9	0.10	0.20	0.40	1.00	1.99	3.99
	Flupyradifurone	11.2	22.5	45.0	0.01	0.01	0.02	0.06	0.12	0.24
	Spiroxamine	131.3	262.5	525.0	0.42	0.84	1.68	4.20	8.40	16.80
NL	Lambda-cyhalothrin	2.5	5.0	10.0	0.02	0.05	0.09	0.23	0.47	0.93
	Esfenvalerate	2.3	4.7	9.3	0.04	0.08	0.16	0.39	0.78	1.57
	Pirimicarb	125.0	250.0	500.0	0.40	0.80	1.60	4.00	8.00	16.00
	Prochloraz	212.3	424.5	849.1	1.36	2.72	5.43	13.58	27.17	54.34
	Bixafen	44.0	88.0	176.0	0.20	0.40	0.80	1.99	3.98	7.97
DK	Glyphosate	576.3	1152.6	2305.1	1.84	3.69	7.38	18.44	36.88	73.76
	Esfenvalerate	2.3	4.7	9.3	0.04	0.08	0.16	0.39	0.78	1.57
	Pendimethalin	239.3	478.5	957.0	0.77	1.53	3.06	7.66	15.31	30.62
AR	Glyphosate	169.8	339.6	679.3	0.54	1.09	2.17	5.43	10.87	21.74
	Fluxapyroxad	10.0	20.0	40.0	0.03	0.06	0.13	0.32	0.64	1.28
	Azoxystrobin	40.0	80.0	160.0	0.13	0.26	0.51	1.28	2.56	5.12
	Lambda-cyhalothrin	3.1	6.3	12.5	0.01	0.02	0.04	0.10	0.20	0.40
	Bixafen	21.9	43.8	87.5	0.07	0.14	0.28	0.70	1.40	2.80

Table A3: Pesticide mixture selection per CSS for soil microbial activity.

CSS	Pesticides	mg/kg MEC	mg/kg PEC	mg/kg 5PEC
ES	Metrafenone	0.010	0.39	1.97
	Propamocarb (hydrochloride)	0.158	8.66	43.3
	Pendimethalin	0.032	2.13	10.6
	Boscalid	0.018	0.40	1.98
	Tebuconazole	0.341	0.18	0.92
PT	Metrafenone	0.108	0.39	1.97
	Boscalid	0.175	0.40	1.98
	Fludioxonil	0.073	0.28	1.42
	Penconazole	0.017	0.13	0.62
	Tebuconazole	0.015	0.18	0.92
FR	Metrafenone	0.041	0.39	1.97
	Boscalid	0.028	0.40	1.98
	Glyphosate	0.257	5.76	28.8
	Zoxamid	0.043	0.38	1.91
	Ametoctradin	0.045	0.34	1.69
CH	AMPA	0.344	2.03	10.1
	Difenoconazole	0.009	0.13	0.67
	Methoxyfenozide	0.009	0.048	0.24
	Tebuconazole	0.007	0.18	0.92
IT	Metrafenone	0.026	0.39	1.97
	Boscalid	0.018	0.40	1.98
	Pendimethalin	0.035	2.13	10.6
	Propamocarb (hydrochloride)	0.089	8.66	43.3
	Tebuconazole	0.013	0.18	0.92
HR	Boscalid	0.134	0.40	1.98
	Tebuconazole	0.075	0.1	0.92
	Phosmet	0.006	0.57	2.85
	Glyphosate	0.167	5.76	28.8
	Dimethomorph	0.006	1.77	8.87
SI	AMPA	0.070	2.03	10.18
	Tebuconazole	0.007	0.18	0.92
	Terbuthylazine	0.009	1.12	5.62
	Thiencarbazone-methyl	0.043	0.060	0.3
CZ	Metrafenone	0.007	0.39	1.97
	Boscalid	0.018	0.40	1.98
	Tebuconazole	0.028	0.18	0.92
	Dimoxystrobin	0.017	0.027	0.13
	Flupyradifurone	0.007	0.12	0.62
NL	AMPA	0.094	2.03	10.1
	Boscalid	0.018	0.40	1.98
	Fluoxastrobin	0.006	0.24	1.21
	Metobromuron	0.183	2.66	13.3
	Prochloraz BTS 44595	0.014	0.021	0.10
DE	AMPA	0.115	2.03	10.1
	Boscalid	0.008	0.40	1.98
	Pendimethalin	0.049	2.13	10.6
AR	AMPA	1.325	2.03	10.1
	Glyphosate	0.545	5.76	28.8
	Methoxyfenozide	0.012	0.048	0.24

Table A4: Pesticide mixture selection per CSS for terrestrial plants.

CSS	Pesticides	MEC	mg/kg PEC	5PEC
ES	AMPA	0.114	2.03	10.1
	Glyphosate	0.083	5.76	28.8
	Metazachlor	0.135	1.33	6.66
	Propyzamide	0.017	2.00	10
	Difenoconazole	0.020	0.13	0.67
PT	Glyphosate	0.793	5.76	28.8
	AMPA	1.910	2.03	10.1
	Iprovalicarb	0.174	0.39	1.99
	Penconazole	0.017	0.14	0.70
	Metolachlor oxanilic	0.037	0.40	2.00
FR	AMPA	0.871	2.03	10.1
	Glyphosate	0.257	5.76	28.8
	Fenbuconazole	0.013	0.46	2.33
	Metrafenone	0.041	0.39	1.97
	Difenoconazole	0.010	0.13	0.67
CH	AMPA	0.344	2.03	10.1
	Difenoconazole	0.009	0.13	0.67
	Myclobutanil	0.006	0.67	3.36
	Emamectin	0.005	0.33	1.65
	Tebuconazole	0.007	0.18	0.92
IT	Propyzamide	0.010	2.00	10.0
	Pendimethalin	0.035	2.13	10.6
	Lambda Cyhalothrin	0.006	0.033	0.16
	Oxyfluorfen	0.046	1.92	9.6
	Difenoconazole	0.009	0.13	0.67
HR	Glyphosate	0.167	5.76	28.8
	AMPA	0.516	2.03	10.1
	Deltamethrin	0.036	0.022	0.11
	Trifloxystrobin	0.010	0.50	2.50
	Tebuconazole	0.075	0.18	0.92
SI	Metolachlor (S)	0.041	1.92	9.60
	Terbuthylazine	0.009	1.12	5.62
	Thiencarbazone-methyl	0.044	0.060	0.3
	AMPA	0.070	2.03	10.1
	Terbuthylazine desethyl	0.006	0.32	1.62
CZ	AMPA	0.135	2.03	10.1
	Diflufenican	0.011	0.25	1.25
	Mecoprop P	0.015	1.60	8.00
	Dimethenamid (P)	0.021	1.15	5.76
	Deltamethrin	0.026	0.022	0.11
NL	Metribuzin	0.040	1.40	7.00
	Metobromuron	0.183	2.66	13.3
	AMPA	0.094	2.03	10.1
	Diflufenican	0.005	0.25	1.25
	Prosulfocarb	0.063	5.33	26.6
DK	AMPA	0.115	2.03	10.1
	Diflufenican	0.018	0.25	1.25
	Fluopyram	0.008	0.26	1.30
	Pendimethalin	0.049	2.13	10.6
	Boscalid	0.008	0.39	1.98
AR	Glyphosate	0.545	5.76	28.8
	AMPA	1.325	2.03	10.1
	Diflufenican	0.007	0.25	1.25
	2,4-D (free)	0.019	0.75	3.75
	Lambda-Cyhalothrin	0.006	0.033	0.16