Disclaimer: This report is part of a project that has received funding by the European Union's Horizon 2020 research and innovation program under grant agreement number 862568.

Ecotoxicological assessment of pesticide mixtures on aquatic non-target species

Abrantes et al.

Deliverable number: 4.2
Due date: 31.08.2024 (month 48)
Dissemination level: PUBLIC
Lead beneficiary: UAVR
Deliverable type: REPORT
Version: 1.0

Disclaimer: This report is part of a project that has received funding by the European Union's Horizon 2020 research and innovation program under grant agreement number 862568.

PROJECT INFORMATION	
Project Title	Sustainable Plant Protection Transition; A global health approach
Project Acronym	SPRINT
Call Identifier	H2020-SFS-2019-2; Sustainable Food Security
Grant Agreement no.	862568
Starting Date	01-09-2020
End Date	31-08-2025
Project duration	60 months
Website address	www.sprint-h2020.eu
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REPORT INFORMATION	
Report Title	Ecotoxicological assessment of pesticide mixtures on terrestrial non-target species
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Deliverable Number	4.2
Work Package	4
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Report Due Date	31.08.2024 (month 48)
Report publish date	31.08.2024 (month 48)

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1 Introduction

Pesticides, including herbicides, insecticides, and fungicides, are widely used in agriculture to protect crops from pests and diseases, thereby boosting crop yields. Their application often involves multiple pesticides, resulting in complex mixtures in the environment (Alaoui et al., 2024; Carvalho, 2017; Silva et al., 2019; Stehle and Schulz, 2015a; Tang et al., 2021). While crucial for food production, these compounds/mixtures raise significant environmental and health concerns, as they may pose severe risks to non-target terrestrial and aquatic ecosystems (Navarro et al., 2024; Nowell et al., 2018; Panico et al., 2022; Schäfer et al., 2011).

Freshwater ecosystems are particularly vulnerable to pesticide contamination, especially if in close proximity to agricultural lands. Pesticides reach water bodies through rainfall, irrigation, and leaching, introducing complex mixtures of chemicals into rivers, lakes, and streams, often at concentrations that can pose serious risks to aquatic life (Adriaanse et al., 2017; Navarro et al., 2024; Stehle and Schulz, 2015b; Suciu et al., 2020). The specific mode of action of these contaminants determines which groups of non-target organisms—such as microorganisms, microalgae, macrophytes, invertebrates, or fish—are primarily affected (Nowell et al., 2018; Schäfer et al., 2010; Schäfer et al., 2011). These chemicals can affect various levels of biological organization, from molecular to entire ecosystems.

The mixtures of pesticides in aquatic systems are dynamic and pesticide residues can interact with each other, leading to additive, synergistic, or antagonistic effects that are difficult to predict (Deneer, 2000; Weisner et al., 2021). These interactions can exacerbate the toxicity of individual compounds, making the overall impact on aquatic organisms more severe than anticipated (Deneer, 2000; Schäfer et al., 2010; Schäfer et al., 2011). Ecotoxicological effects of pesticide mixtures can occur at concentrations considered safe for individual chemical exposures (Panico et al., 2022). Therefore, the impact of pesticide mixtures on aquatic systems can be both profound and multifaceted.

Current regulatory risk assessments (EFSA PPR, 2013) focus on evaluating the toxicity of individual active ingredients, which may not accurately represent real-world scenarios where multiple pesticide residues coexist. This approach likely underestimates the potential risks of pesticide mixtures (Schäfer et al., 2010; Weisner et al., 2021). The risk from exposure to chemical mixtures can be evaluated either through direct testing of the real or prepared mixtures (whole-mixture approach) or by using mathematical predictions

based on the individual components (components-based approach) (Boop et al., 2018; EFSA, 2019). Predicting the response of organisms exposed simultaneously to pesticide mixtures is one of the major current challenges in ecotoxicological risk assessment.

To address this challenge, and within the goals of SPRINT work package four (WP4), a key focus is on testing the ecotoxicity of pesticide mixtures on aquatic organisms. The testing strategy selected for the SPRINT project utilized a whole-mixture approach, involving the ecotoxicological testing of mixtures prepared in laboratory, in different aquatic organisms, encompassing pelagic and benthic organisms. Furthermore, a **tiered approach**, from simple assessments at low-tier (current Deliverable – 4.2) to more complex assessments at high-tier (Deliverable 4.3 in preparation), was implemented based on the EFSA guidelines for combined exposure to multiple chemicals (EFSA, 2019).

The selection of pesticide mixtures was determined through a prioritization process that incorporated the results of the SPRINT survey, conducted in 2021 at the eleven case studies sites (CSS), as well as individual pesticides' risk quotient, drawing upon the EU regulatory risk assessment approach of the European Food Safety Authority (EFSA PPR, 2013) (Abrantes at al under review; see more information in chapter 2).

The ecotoxicological effects were assessed not only on current EFSA organisms and tests, but also on alternative (native) species, emphasizing indirect and chronic effects. By doing so, SPRINT introduces a more comprehensive approach for evaluating the effects of pesticide mixtures.

The overview of all tests conducted during 2022–2024 is presented in Table 1.1. Chapter 2 outlines the procedures used to prioritize and select the 11 mixtures for aquatic testing. Chapters 3 through 9 describe the methodologies, results, discussions, and conclusions for each individual test. The final chapter, Chapter 10, summarizes the key findings, discusses implications for policy and regulation, and provides a future outlook

Table 1.1 Overview of all aquatic ecotoxicology tests performed during 2022 – 2024. 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**
Freshwater algae *(Raphidocelis subcapitata*	Growth inhibition	96h	11	CTR, MEC, PEC, 3PEC, 5PEC	6	In preparation	UAVR	3
Freshwater plant *Lemna minor*	Growth inhibition	7 days	11	CTR, MEC, PEC, 3PEC, 5PEC	3	In preparation	UAVR	4
Freshwater crustacean *Dahnia magna**	Immobilization	48h	11	CTR, MEC, PEC, 3PEC, 5PEC	4	In preparation	UAVR	5
Freshwater insect *Chironomus riparious*	Immobilization Gene expression[a,b] Enzimatic activity[a,b]	48h	11 3 3	CTR, MEC, PEC, 3PEC, 5PEC	4	In preparation	UAVR	6
Fish *Danio rerio*	Lethality Behaviour[b] Cholinesterase activity (ChE)[b] Gene expression[a,b]	4	11 11 11 3	CTR, MEC, PEC, 3PEC, 5PEC	6 6 3 9	In preparation	UAVR	7
Native freshwater crustacean *Daphnia longispina*[a, b]	Immobilization[a,b] Reproduction[a,b]	48h 21 days	11 9	CTR, MEC, PEC, 3PEC, 5PEC	4 10	In preparation	UAVR	8 9

[a.] Additional test performed that were not included in the GA.
[b.] New SPRINT indicators.

2 Selection of the pesticide mixtures for testing

The methodology for identifying priority pesticides for mixture formulation involved a prioritization process based on the SPRINT survey conducted in 2021 across eleven case study sites (CSS), considering their individual risk quotients and frequencies of occurrence at each site. Briefly, per CSS the pesticides were ranked based on their frequency of occurrence in water samples and their risk quotient (RQ), calculated as the ratio between toxicity and exposure (RQ= Predicted Environmental Concentration (PEC) /Regulatory Acceptable Concentration (RAC) (see 2.2, 2.3. 2.4 and 2.5). The normalized RQs were multiplied by the normalized frequency of occurrence in water to give the final score for each pesticide (2.5). The five pesticides with the highest scores in each CSS were selected for performing the ecotoxicological tests. The methodology involved the following steps:

2.1 Pesticides distribution in aquatic ecosystem

The pesticide selection was based on the pesticides quantified in the 64 surface water bodies sampled from 11 CSS (10 in Europe and 1 in Argentina) between Spring and Autumn 2021. Of the 115 residues detected in water samples across all CSS (Navarro et al., 2024), only 83 were above the limit of quantification. Only those approved by the European Union (excluding Argentina) and those with available ecotoxicological information were included in the pesticide prioritization methodology.

2.2 Predicted Environmental Concentration in water (PECw)

The PECw value for each of the selected pesticide were sourced from the EFSA report, step 1. If the PECw value was not available in those reports, they were calculated using the FOCUS (FOrum for Coordination of Pesticides Fate Models and Their Use) simulation model steps 1-2. The variables considered in the PEC calculation for each pesticide include molecular weight, water solubility at 20°C, soil degradation DT_{50} (time for the concentration to decline to 50% of the amount at application), soil sorption coefficient (K_{oc}), rate of chemical decomposition in water-sediment systems ($DT_{50sediment/water}$), typical number of pesticide applications, application interval, and application rate for the CSS-dominant crop.

2.3 Ecotoxicological data collection on pesticides

Data on toxicity and exposure (ecotoxicological information) on all the pesticides found in water samples from the SPRINT CSS were extracted from the Pesticides Properties database (PPDB) (Lewis et al., 2016). We gathered all available information on median Lethal Concentrations (LC_{50}) and median Effect Concentrations (EC_{50}) for aquatic species from short-term tests, as well as No Observed Effect Concentrations (NOEC) from long-term tests. We also recorded detailed notes on the test procedures to identify any non-standard methods and support decisions regarding the inclusion of these benchmarks in subsequent steps.

2.4 Regulatory Acceptable Concentration (RAC) for the aquatic organisms

Following the guidelines specifically designed for pesticide risk assessment in edge-of-field surface waters, as outlined in the EFSA PPR (2013) document, we calculated Regulatory Acceptable Concentrations (RACs). These calculations were performed in accordance with Regulation (EC) No. 1107/2009 and the referenced Annex VI to Directive 91/414/EEC. RACs were established as ecological thresholds that allow for only negligible effects on populations, ensuring a high level of environmental protection in line with regulatory requirements. The RACs were calculated based on the retrieved ecotoxicological information and appropriate Assessment Factors (AF). This involved normalizing ecotoxicological endpoints, specifically EC_{50}/LC_{50} for acute effects and NOEC or EC_{10} for chronic effects, using defined AF. As per the guidelines in Table 1-3 of the EFSA PPR (2013) document, we applied an AF of 100 for acute effect assessments and 10 for chronic effect assessments. In the present context to support the ranking of pesticides for selecting mixtures to undergo ecotoxicity testing, we focused on calculating only the most conservative TIER 1- RAC for each PPP. This was done by normalizing either the lowest LC_{50}/EC_{50} (for RAC_{acute}) or the lowest NOEC (for $RAC_{chronic}$) found on the respective database, using the appropriate AF. This approach ensures a cautious and protective stance in our assessment, aligning with the goal of identifying the most potentially impactful substances for further testing.

2.5 Pesticides ranking

A relevant pesticide mixture for each crop-specific scenario (CropSS) for the water compartment was defined as a combination of up to five pesticides of concern. These pesticides were selected based on two primary criteria (Figure 2.1):

Criterion A: Risk Quotient of each pesticide for aquatic organisms (RQ), which was calculated as the ratio between the PEC and the RAC. The higher the RQ value obtained per pesticide the higher its risk.

Criterion B: Frequency of occurrence of each pesticide in the aquatic system sampled per CSS (values given in percentage).

For each criterion, we normalized the scores to a scale of 0 to 1. We then ranked the pesticides by multiplying their risk quotient scores by their frequency of occurrence scores. To determine the testing mixture for each CSS, we selected the top 5 pesticides (Table 2.1) that achieved the highest combined scores from this multiplication process.

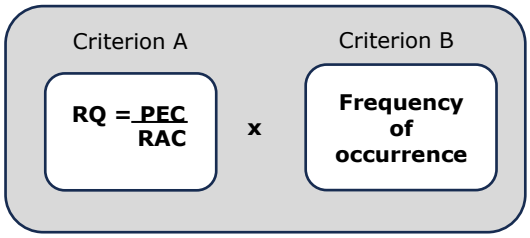

Figure 2.1. EFSA approach followed to rank and select the pesticides that composed each mixture aquatic ecosystems.

2.6 Concentrations tested on the ecotoxicological

After the selection of the substances for each pesticide mixture, the ecotoxicological tests were performed using 5 concentrations (Table 2.1):

- Measured Environmental Concentration (MEC)- field concentration (median)
- Predicted Environmental Concentration (PEC)
- Three times higher concentration than PEC (3PEC)
- Five times higher concentration than PEC (5PEC)
- Control (CTR)

While concentrations were confirmed through chemical analysis, all results are presented using nominal concentrations. Specifically, the concentrations are expressed as MEC, PEC, 3PEC and 5PEC. This approach of using nominal concentrations for reporting ensures consistency and clarity in presenting the test results, despite the verification of actual concentrations through chemical analysis.

Table 2.1. Pesticides mixtures selected per Country/CSS for the aquatic ecotoxicological assessment, sorted from higher to lower RQ coefficient.

Country	CSS	Pesticides	MEC (µg/l)	PEC (µg/l)	3PEC (µg/l)	5PEC (µg/l)
Spain ES	**CSS1**	Chlorantraniliprole	0.0076	34.83	104.5	174.3
		Fludioxonil	0.0135	14.20	42.60	71.00
		Azoxystrobin	0.0151	110.8	332.4	554.0
		Fluopicolide	0.0130	103.8	311.4	519.0
		Acetamiprid	0.0246	4.970	14.9	24.9
Portugal PT	**CSS2**	Terbuthylazine	0.0052	243.5	730.5	1,218
		Dimethomorph	0.1098	243.4	730.3	1,217
		Ethofumesate	0.2303	297.7	893.2	1,489
		Tebuconazole	0.0615	1.600	4.800	8.000
		Glyphosate	0.1550	104.8	314.4	524.1
France FR	**CSS3**	Chlorantraniliprole	0.1002	34.83	104.5	174.2
		Zoxamide	0.0291	123.3	369.8	616.3
		Fluopicolide	0.2885	103.8	311.4	519.0
		Trifloxystrobin	0.0075	13.47	40.41	67.35
		Fluopyram	0.0294	20.73	62.19	103.7
Switzerland CH	**CSS4**	Terbuthylazine	0.0052	243.5	730.5	1,218
		Ethofumesate	0.0770	297.7	893.2	1,489
		Tebuconazole	0.0169	1.600	4.800	8.000
		Iprovalicarb	0.0431	273.1	819.4	1,366
		Glyphosate	0.1380	104.8	314.4	524.1
Italy IT	**CSS5**	Terbuthylazine	0.0042	243.5	730.5	1218
		Azoxystrobin	0.0182	110.8	332.4	554.0
		Glyphosate	3.4990	104.8	314.4	524.1
		Spinetoram	0.1320	0.7840	2.352	3.920
		Acetamiprid	0.0816	4.967	14.91	24.85

Fungicide
Herbicide
Insecticide

Table 2.1. (Cont.) Pesticides mixtures per Country/CSS for the aquatic ecotoxicological assessment.

Country	CSS	Pesticides	MEC (µg/l)	PEC (µg/l)	3PEC (µg/l)	5PEC (µg/l)
Croatia HR	**CSS6**	Terbuthylazine	0.0006	243.5	730.5	1218
		Glyphosate	0.0084	104.8	314.4	524.1
		Trifloxystrobin metabolite CGA 321113	0.0008	145.3	436.0	726.7
		AMPA	0.0030	40.90	122.7	204.5
Slovenia SL	**CSS7**	Terbuthylazine	0.0025	243.5	730.5	1,218
		Azoxystrobin	0.0137	110.8	332.4	554.0
		Fludioxonil	0.0096	14.20	42.60	71.00
		Mecoprop (P)	0.0201	400.1	1,200	2,001
		Glyphosate	0.0820	104.8	314.4	524.1
Czech Republic CZ	**CSS8**	Terbuthylazine	0.1711	243.5	730,5	1218
		Fluopicolide	0.0222	103.8	311,4	519.0
		Fludioxonil	0.1413	14.20	42.60	71.00
		Fenhexamid	0.1216	312.0	936.0	1,560
		Tebuconazole	0.0346	1.600	4.800	8.000
Netherlands NL	**CSS9**	Terbuthylazine	0.0379	243.5	730.5	1,218
		Azoxystrobin	0.1606	110.8	332.4	554.0
		Tebuconazole	0.0424	1.600	4.800	8.000
		Dimethenamid (P)	0.1268	243.4	730,2	1,217
		Metobromuron	0.0757	568.1	1,704	2,840
Denmark DK	**CSS10**	Glyphosate	0.1940	104.8	314.4	524.1
		Fluopyram	0.0052	20.70	62.10	103.5
		Metalaxyl Metabolite CGA	0.0175	18.30	54.90	91.50
Argentina AR	**CSS11**	Cyproconazole	0.0313	24.52	73.56	122.6
		Metribuzin	0.0491	9.700	29.10	48,50
		2,4-D	0.2699	470.7	1,412	2,354
		MCPA	0.1449	1.350	4.050	6.750
		Glyphosate	0.1940	104.8	314.4	524.1

Fungicide
Herbicide
Insecticide

3 Growth inhibition test with the microalgae *Raphidocelis subcapitata*

3.1 Introduction

The test followed the OECD 201 (2006) standard guideline with adaptations to use 24-well plates (Geis et al., 2000), hence reducing the volume to avoid the use of high volumes of waste pesticides. The objective of the test was to quantify the effects of pesticides mixture on the *R. subcapitata* growth over the exposure period.

3.2 Methodology

The microalgae were exposed during 96h to four different pesticides mixtures concentrations: MEC (Measured Environmental Concentration), PEC (Predictable Environmental Concentration), 3PEC and 5PEC and the control (MBL Medium). Assays were performed using 24 multi-well plates containing 900µL of the tested solutions or MBL medium used as control. Six replicates were made for each treatment and 100µL of the microalgae culture (105 cells/mL) were added in each well (Figure 3.1). The test was conducted in an incubator chamber under controlled temperature conditions (23±2°C), light intensity (4440 – 6500 lux) and without photoperiod (24hL). The test endpoint, growth rate day-1 was determined using the absorbance at the beginning and at the end of the test.

Figure 3.1 Cultures of *Raphidocelis subcapitata* and 24 multi-well test chambers used to carry out the assays.

3.3 Results and Discussion

Results showed that all the eleven tested mixtures inhibit the growth rate of the microalgae (Figure 3.2).

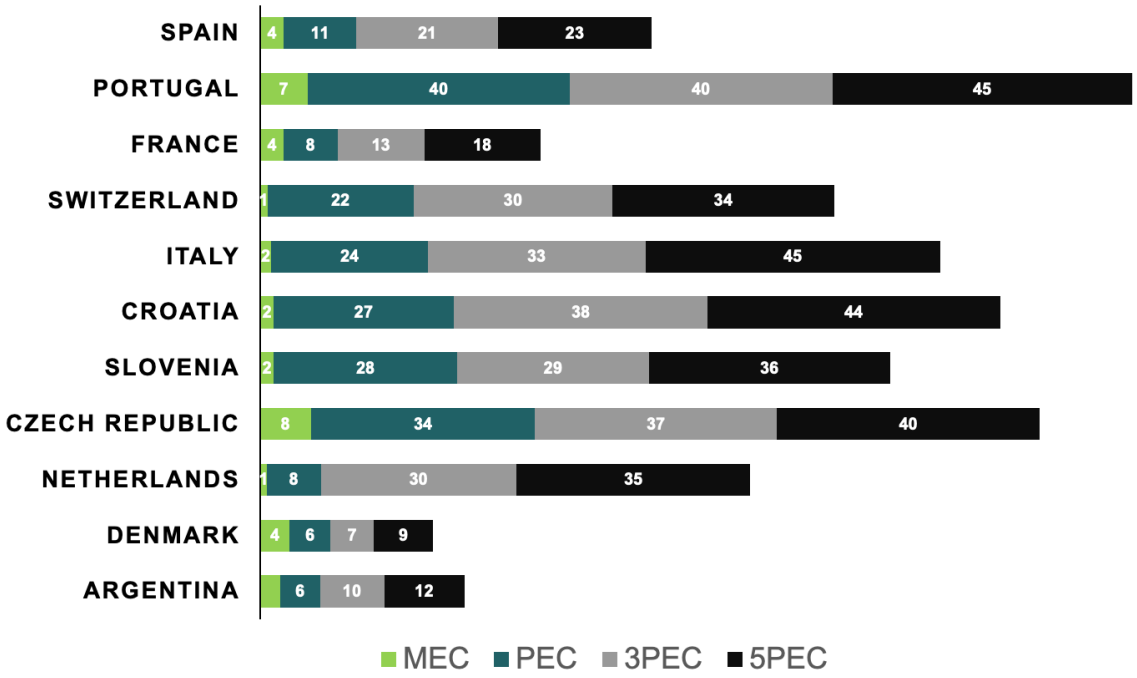

Figure 3.2 Percentage of growth inhibition of *Raphidocelis subcapitata* when exposed to distinct mixtures (percentages displayed refer to average inhibition among replciates). MEC – measured environmental concentration; PEC – predicted environmental concentration.

For certain CSS mixtures, PEC, 3PEC, and 5PEC concentrations induced expressive inhibitory effects, with growth inhibition exceeding 30%. This was observed for pesticide mixtures from Portugal, Italy, Croatia, and the Czech Republic. Conversely, pesticide mixtures from Spain, France, Denmark, and Argentina exhibited only slight to moderate growth inhibition, with growth inhibition values below 25%. Importantly, none of the pesticide mixtures caused significant growth rate inhibition at the MEC concentrations, with growth inhibition values below 8% for all pesticide mixtures.

3.4 Conclusions

- At MEC concentrations, none of the pesticide mixtures resulted in significant growth rate inhibition.
- PEC, 3PEC, and 5PEC concentrations of certain CSS pesticide mixtures led to significant growth inhibition in *Raphidocelis subcapitata*, with inhibition exceeding 30%. This was observed particularly for mixtures from Portugal, Italy, Croatia, and the Czech Republic.
- Pesticide mixtures from Spain, France, Denmark, and Argentina caused only slight to moderate growth inhibition.

4 Growth inhibition test with the macrophyte *Lemna minor*

4.1 Introduction

The test adhered to the OECD 221 (2006) guidelines, with modifications to reduce waste pesticide volumes (Kaza et al., 2007). Its main objective was to evaluate the impact of the eleven different pesticide mixtures (1 per CSS) on the *L. minor* growth over the seven days of exposure period based on the frond number and dry weight.

4.2 Methodology

The macrophyte was exposed during 7 days to four different pesticides mixtures concentrations: MEC (Measured Environmental Concentration), PEC (Predictable Environmental Concentration), 3PEC and 5PEC and the control (Steinberg Medium). Assays were performed using 6-multi-well plates containing 10mL of the tested solutions. Three replicates were established for each treatment and 9 plants with colonies 3 visible fronds were randomly transferred to each well (Figure 4.1). The test was conducted in an incubator chamber under controlled temperature conditions (23±2°C), light intensity (5000-6500 lux) and without photoperiod (24h^{L}). The test endpoint, growth rate and percentage of growth inhibition, were determined using the frond number and dry weight at the beginning and at the end of the test.

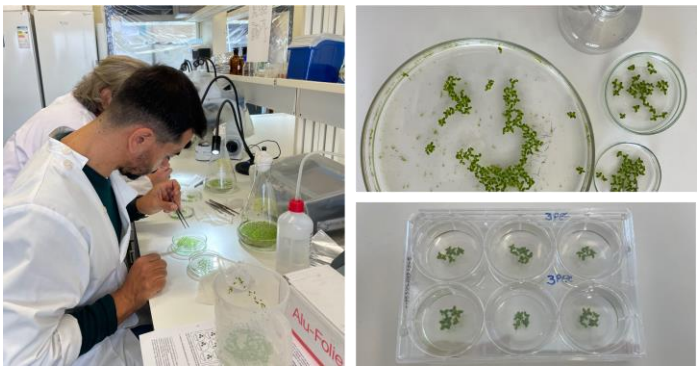

Figure 4.1 Colonies of *Lemna minor* and multi-well test chambers used to carried out the assays.

4.3 Results and Discussion

The pesticides mixtures for all countries induced *Lemna minor* growth inhibition, with statistical differences after PEC, 3PEC and 5 PEC exposure (Figure 4.2).

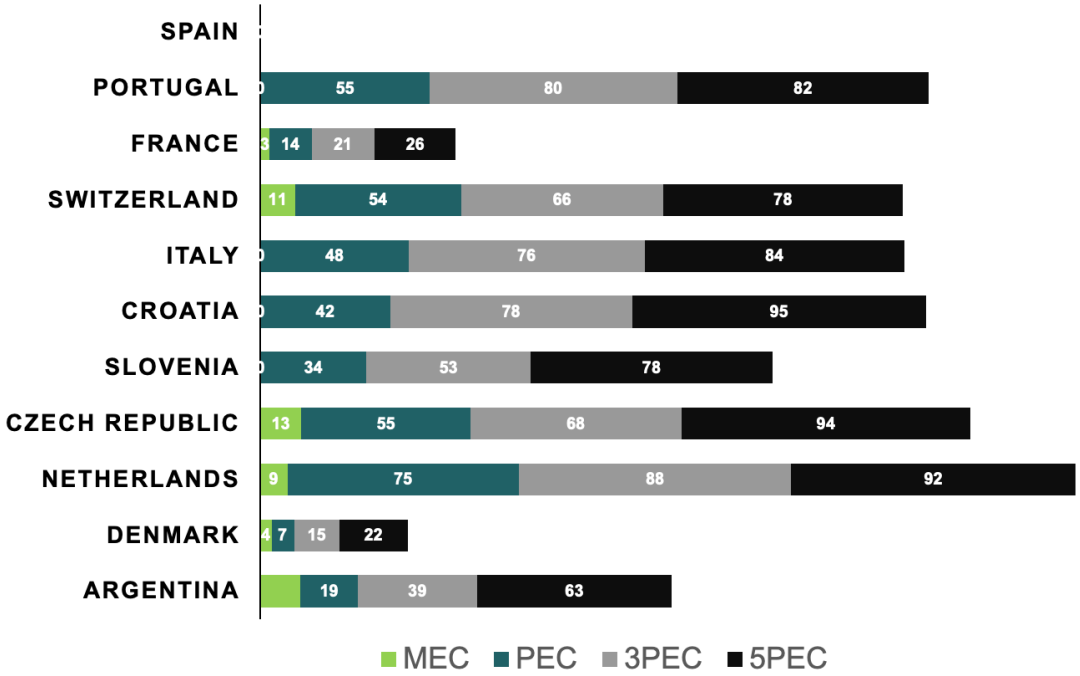

Figure 4.2 Percentage of growth inhibition of *Lemna minor* when exposed to distinct mixtures. MEC – measured environmental concentration; PEC – predicted environmental concentration.

For most of the mixtures tested, growth inhibition after 5PEC and 3PEC exposures was consistently above 60% and 40%, respectively. Exceptions were noted in Denmark (22% and 15%, respectively) and France (26% and 21%, respectively). A similar trend was

observed at PEC exposure, where growth inhibition generally exceeded 34%, except in Denmark (7%), France (14%), and Argentina (19%). In Spain, pesticide mixtures did not affect *Lemna minor* growth at any concentration, likely due to the absence of herbicides in the mixtures. For MEC concentrations, growth inhibition remained below 13% across all countries, with no statistically significant differences from the control.

4.4 Conclusions

- The pesticide mixtures from Portugal, Switzerland, Italy, Croatia, Slovenia, the Czech Republic, The Netherlands, and Argentina exhibit values for PEC, 3PEC, and 5PEC that lead to an expressive inhibition of *Lemna minor* growth.
- In opposite, the pesticide mixtures from France, and Denmark result in growth inhibition percentages of less than 25% for *L. minor*. In the case of Spain, no toxicity was observed at any concentrations.
- The MEC concentrations in general slightly affected the *L. minor* growth, with maximum values of inhibition recorded in Argentina and the Czech Republic (13%).

5 Acute immobilization test with *Daphnia magna*

5.1 Introduction

In the acute immobilization test the effects on the swimming capability of *D. magna* neonates (<24h) were tested. *D. magna* (Figure 5.1) has been used as a standard organism where the percentage of daphnids that were immobilized (being no longer capable of swimming) were evaluated after 48 hours of pesticides exposure. The test follows the OECD 202 (2004) guideline.

5.2 Methodology

The swimming capability of *D. magna* neonates (<24h) were evaluated after 48h of exposure, at 18-22ºC, in the dark and with no food. Twenty animals, divided into four groups of five animals each were used at each test concentration (MEC, PEC, 3PEC and 5PEC) and controls.

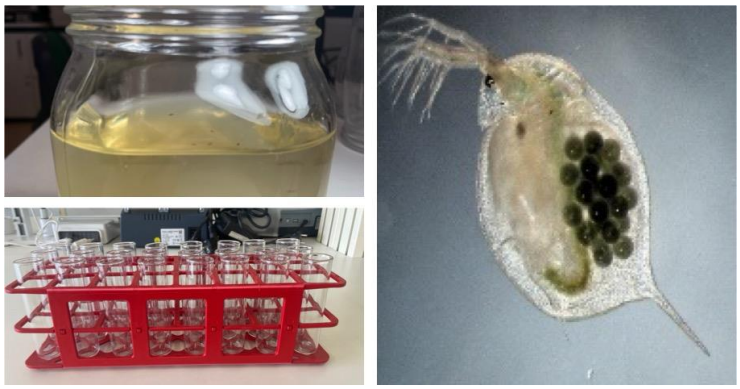

Figure 5.1 Cultures of *Daphnia magna* and test tubes used to carry out the assays.

5.3 Results and Discussion

Pesticide mixtures from Spain, France, Switzerland, and Slovenia exhibited very high immobilization rates at PEC, 3PEC, and 5PEC exposures, with values of 40%, 80%, and 90%, respectively (see Figure 5.2). Additionally, the Netherlands' pesticide mixture also showed elevated immobilization, at 85%, after 5PEC exposure. In contrast, no significant effects were observed for Argentina, Denmark and Portugal. Exposure to MEC concentrations did not induce any immobilization of *D. magna* in any of the mixtures tested. While in Spain, none of the tested concentrations (PEC, 3PEC, and 5PEC) caused growth inhibition in *L. minor*. However, significant effects were observed for *D. magna*, with immobilization rates of 85%, 100%, and 100%, respectively, at these concentrations.

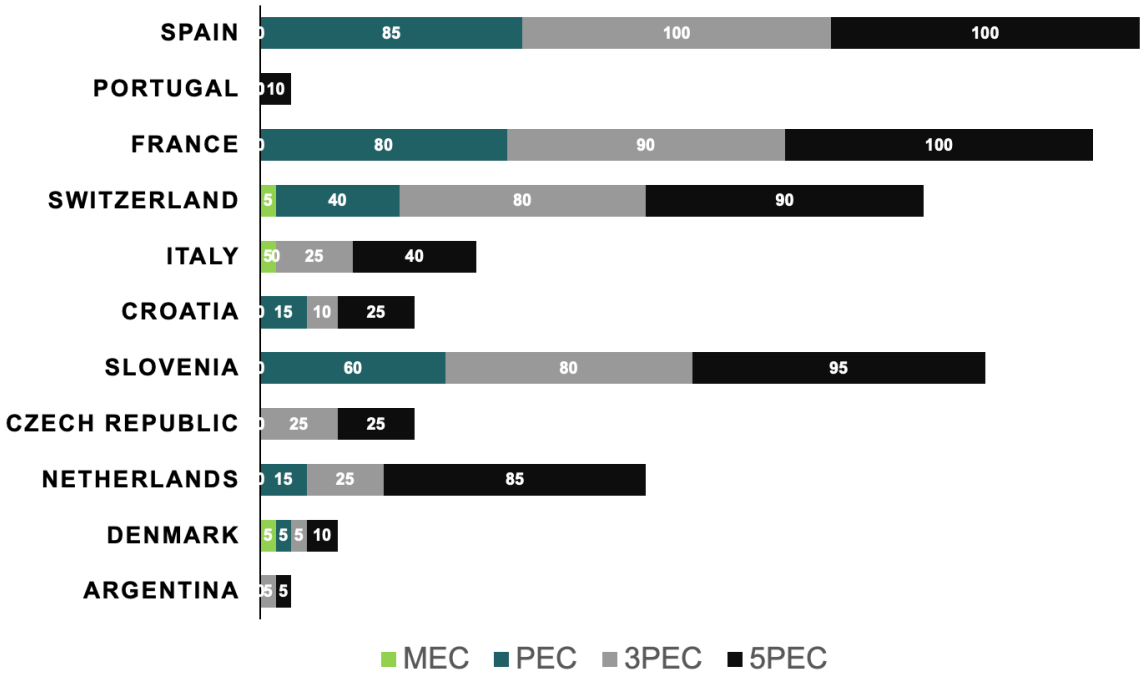

Figure 5.2 Percentage of immobilization of *Daphnia magna* when exposed to distinct mixtures. MEC – measured environmental concentration; PEC – predicted environmental concentration.

5.4 Conclusions

- Pesticide mixtures from Spain, France, Switzerland, and Slovenia caused very high immobilization rates in *D. magna* at PEC, 3PEC, and 5PEC exposures, with rates of 40%, 80%, and 90%, respectively. The Netherlands also showed elevated immobilization after 5PEC exposure.
- No significant effects on *Daphnia magna* were observed for pesticide mixtures from Argentina, Denmark, and Portugal.
- Additionally, MEC concentrations did not induce immobilization in any of the mixtures tested.

6 Acute Immobilization test with *Chironomus riparius*

6.1 Introduction

In the acute immobilization test, the effects on the mobility of first instar larvae (<24 hours after egg hatching) of *C. riparius* were assessed. *C. riparius* (Figure 6.2) is a standard test organism, and the percentage of larvae that became immobilized (unable to move) was evaluated after 48 hours of pesticide exposure. This test follows the OECD 235 (2011) guideline.

6.2 Methodology

I instar larvae of *C. riparius* were exposed during 48h to four different concentrations: MEC, PEC, 3PEC and 5PEC and control (ASTM Medium). Assays were performed using crystal vessels containing 10 mL of the tested solutions or ASTM medium. Four replicates were used for each treatment and 5 larvae were added to each vessel (Figure 6.1). The test was conducted in a chamber under controlled temperature conditions (20±2°C), and photoperiod (16:8h). The test endpoint, immobilization, was determined by optical observation using a stereo microscope and a Pasteur pipette. In addition to immobility, any abnormal behaviour or unusual appearance was reported. The O_2 dissolved and pH were measured at the beginning and the end of the tests.

Figure 6.1 Test tubes used to carry out the assays with *Chironomus riparius*.

In addition to the standard immobilization endpoint, additional efforts beyond what was initially agreed upon were undertaken to gain a deeper understanding of the impacts of PPP mixtures on sub-individual parameters. These parameters can indicate effects at higher levels of biological organization, thereby serving as early warning indicators. To this end, three CSS with very distinct mixtures (France, The Netherlands, and Argentina) were selected to evaluate the sub-organismal response (gene expression and enzymatic activity) of the IV instar larvae of *C. riparius*. This stage was used instead of I instar due to possible limitations regarding the amount of RNA obtained. The larvae were exposed (CTR, MEC, PEC, 3PEC and 5PEC) prepared in the ASTM culture medium and maintained for 48h, with aeration and without food. Then, three individual larvae per condition and replicate (3 in total) were collected and frozen at -80°C for the subsequent analysis in the Biology and Ecotoxicology Group in the UNED, Spain. So, 9 larvae per condition for both gene expression and enzymatic activity were employed for the analysis. RNA extraction and cDNA synthesis from these samples were developed at UNED. Then, the response of 14 genes related to endocrine system (*JHAMT, Met, kr-h1, EcR, E93*), detoxification response (*Cyp12a2, Cyp9f2, GSTd6, GSTe2, GSTs1*), oxidative stress (*CAT, SOD CuZn*) and stress response (*hsp10, hsp60*) were analyzed by Real-Time PCR (RT-PCR) including two references genes (*rpL11, rpL13*). Moreover, the enzymatic activity of Phenol oxidase (PO), Glutathione S transferase (GST) and Acetylcholinesterase (AChE) was analyzed. The methodology employed for both kinds of analysis can be consulted Muñiz-González & Martínez-Guitarte (2020). The results at sub-individual level are currently being analyzed.

6.3 Results and Discussion

The results showed significant effects of all pesticide mixtures, except for the Czech Republic, which exhibited a maximum effect of 25% when exposed to 5PEC (Figure 6.2). Notably, Argentina, Slovenia, Croatia, France, and Portugal displayed higher immobilization values in the PEC, exceeding 50%. For the remaining countries, PEC, 3PEC, and 5PEC were highly toxic, with Argentina, Italy, and France experiencing total immobilization (100%). In Portugal and Argentina, even the realistic MEC caused a substantial toxic effect, with 40% and 45% immobilization, respectively.

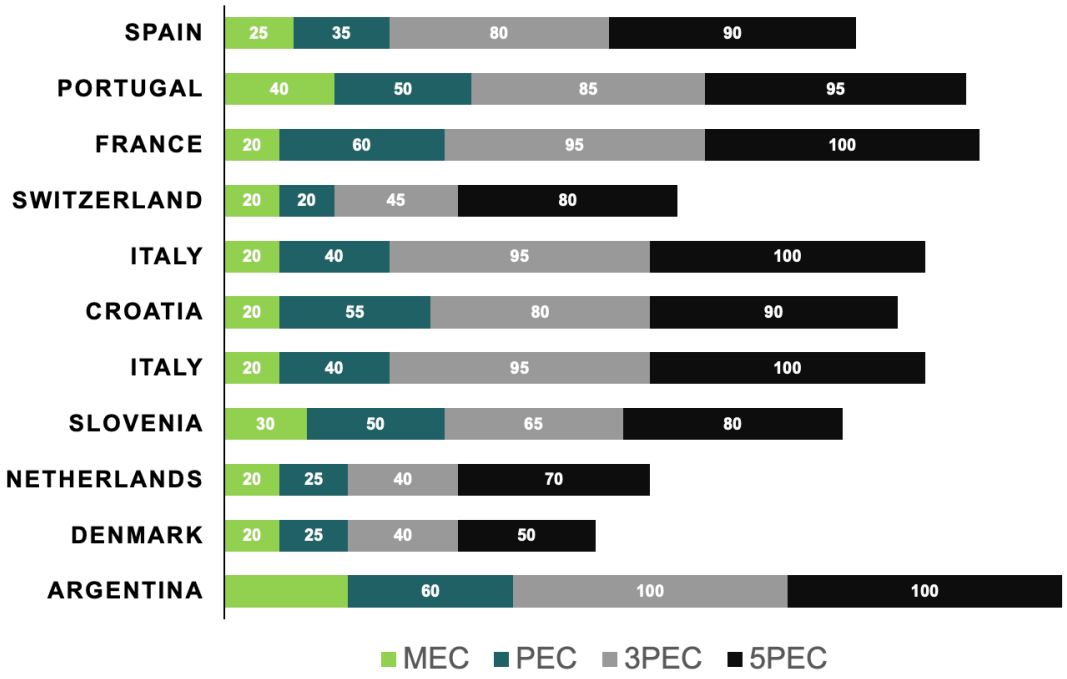

Figure 6.2 Percentage of immobilization of *Chironomus riparius* when exposed to distinct mixtures. MEC – measured environmental concentration; PEC – predicted environmental concentration.

6.4 Conclusions

- All pesticide mixtures, except for the Czech Republic, induced significant effects on *C. riparius* larvae.
- PEC, 3PEC, and 5PEC exhibited high toxicity, leading to total immobilization (100%) in Argentina, Italy, and France.
- In Portugal and Argentina, even the realistic MEC caused considerable toxic effects, resulting in 40% and 45% immobilization, respectively.

7 Fish Embryo Acute Toxicity (FET) Test with the zebrafish *Danio rerio*

7.1 Introduction

The test was carried out following the guideline OECD 236 (2013). This test is a replacement for the originally proposed in the GA standard tests with juvenile or adult fish, as amended and reported in SPRINT D10.1, in compliance with the 3R's (Replacement, Reduction and Refinement) policy of the EU on animal used for scientific purposes (Directive 2010/63/EU). The objective of the test was to quantify the effects of pesticide mixtures on the newly fertilised zebrafish eggs of *D. rerio* over the exposure period, checking the lethality. Every 24 hrs, up to four apical observations were recorded as indicators of lethality: (i) coagulation of fertilised eggs, (ii) lack of somite formation, (iii) lack of detachment of the tail-bud from the yolk sac, and (iv) lack of heartbeat.

7.2 Methodology

Newly fertilised zebrafish eggs of *D. rerio* were exposed during 96h to the 11 mixtures at four concentrations (MEC, PEC, 3PEC and 5PEC) plus zebrafish culture water as control (Figure 7.1). Assays were performed using 24 multi-well plates containing 2mL of the tested solutions or culture medium. Six replicates were established for each treatment and one egg (<24h) was added to each well, (n=36). The test was conducted in an incubator chamber under controlled temperature conditions (26±2°C), and photoperiod (16:8h) within a zebrafish facility. The test endpoint, coagulation of fertilised eggs, (ii) lack of somite formation, (iii) lack of detachment of the tail-bud from the yolk sac, and (iv) lack of heartbeat was determined by optical observation using a stereo microscope. At the end of the exposure period, acute toxicity is determined based on a positive outcome in any of the four apical observations recorded, to determine the mortality percentage (%).

Moreover, cholinesterase (ChE) activity was determined by employing three replicates with 2 independent pools of 5 larvae for each treatment (MEC, PEC, 3PEC and 5PEC) and control. The samples were collected in 2 mL Eppendorf tubes from each country (CSS) after 96h of exposure and maintained at -80 ºC for the subsequent cholinesterase (ChE) activity. Eppendorf was thawed on ice, homogenized with a sonicator (KIKA Labortechnik U2005 Control), and centrifuged (4 ºC, 10000 g, 10 min) to isolate the post-mitochondrial supernatant (PMS). The ChE activity was measured in 96-well microplates (in

quadruplicate) using spectrophotometric methods (Thermo Scientific Multiskan Spectrum, USA) and expressed as nmol/min/mg protein. ChE activity was determined using acetylcholine as substrate measuring the increase in absorbance (at 414 nm) due to the conjugation product between thiocholine (a degradation product of acetylthiocholine) and 5,5-dithiobis-2-nitrobenzoic acid (DTNB) according to Ellman et al. (1961). The activity of ChE was measured in 50 µL PMS and 250 µL reaction mixture (acetylthiocholine (7.5 mM), DTNB (10 mM) in potassium phosphate buffer (0.1 M, pH 7.2).

Finally, the behavioral response after mixture exposure of *D. rerio* larvae was assessed using the ZebraBox-ZEB 478 tracking system (software version 3.22, Viewpoint Life Sciences, Lyon, France) established at the Department of Biology, University of Aveiro, Portugal. The detailed protocol can be consulted in (Franco et al., 2019). For this analysis, six replicates were established for each treatment and one egg (<24h) per well (n=36). To summarise, the locomotion was evaluated for 24 embryos per treatment, including the control, in 24-well plates (one embryo per well) at 120 and 144 hpf (hours post fertilization). The ZebraBox system, which utilizes an infrared camera, was used to monitor and record videos of zebrafish larvae movement at a rate of 25 frames per second. It was observed that zebrafish larvae tend to exhibit less locomotion during periods of light. Light-dark intervals were implemented to stimulate movement using internal LED lights, following the method described by (Andrade et al., 2016). The experiment involved recording the larvae movements for a total of 14 minutes and consisted of two cycles, each comprising 6 minutes of light followed by 2 minutes of darkness. The parameters analyzed were the total swimming distance and the percentage distance in the outer zone. The distances traveled and the total duration of locomotion was separately measured and recorded for each dark and light period in each replicate.

Figure 7.1 Zebrafish facility at the Department of Biology, University of Aveiro.

Additional work beyond what was defined in the grant agreement was conducted to analyze the gene expression of zebra fish to PPP mixtures. Based on the ChE results, three CSSs (Portugal, Switzerland, and Slovenia) were selected for this analysis. Following the same conditions of the OECD 236 (2013), newly fertilised zebrafish eggs of *D. rerio* were exposed for 96h to these 3 mixtures at four concentrations (MEC, PEC, 3PEC and 5PEC) plus a control. Assays were performed using plastic Petri dishes containing 50mL of the tested solutions or culture medium and 100 eggs. After 96h, three pools of 15 larvae were collected for each condition and replicated (3), so 9 pools in total for each condition were maintained at -80 °C for the subsequent analysis. During this year, the protocol for RNA extraction was optimized, in summary, the larvae were homogenized in 500 µL of TRIzol, following the manufacture instructions and then the synthesis of complementary DNA (cDNA) was done for the gene expression analysis by RT-PCR (Muñiz-González & Martínez-Guitarte, 2020). It involved the selection of the genes to analyse, according to the previously published references. optimization of the primers by conventional PCR (final volume of 20 µL) and confirmed the band by electrophoresis using 1 % agarose gel prepared in 1X TBE buffer (Tris-Boric acid-EDTA) and 6µL of gel red dye. The electrophoresis was run at 80 V for 1h. The gel was visualized in a Chemigenius 3 gel documentation system (SynGene – UK). Once the unique band was confirmed, the next step was obtaining the efficiencies of the genes since we will carry out the analysis of the RT-PCR results, by relative quantification. For that, this value is obtained by the slope of the standard curve obtained from serial dilutions (1/2; 1/4; 1/8; 1/16) of PCR products, previously obtained and using a negative (without product) as an internal control (Pfaffl, 2004), employing the same PCR conditions of the experiments following ($R2 > 0.98$ for all primers) and based on the equation: $E=10(-1/slope)-1$. So, 16 genes were selected for the RT-PCR analysis, 2 genes of reference *β-actin* and *eef1a*, then genes related to oxidative stress (*keap 1, nrf2, Cat*), detoxification response (*cyp1a, gsta1*), neurotoxic (*elavl3, syn2a, Ache*), endocrine system (*gh, igf-1, tshβ, dio1*) and apoptosis (*bcl2, caspase9*). The results are currently being processed.

7.3 Results and Discussion

Regarding the zebrafish acute toxicity test, only slight toxicity was observed compared to the former species. Croatia, France, and Spain stood out for their higher toxicity levels, though the maximum immobilization observed was 25% when exposed to 5PEC (Figure

7.2). In contrast, Portugal and The Netherlands showed no immobilization at any tested concentration.

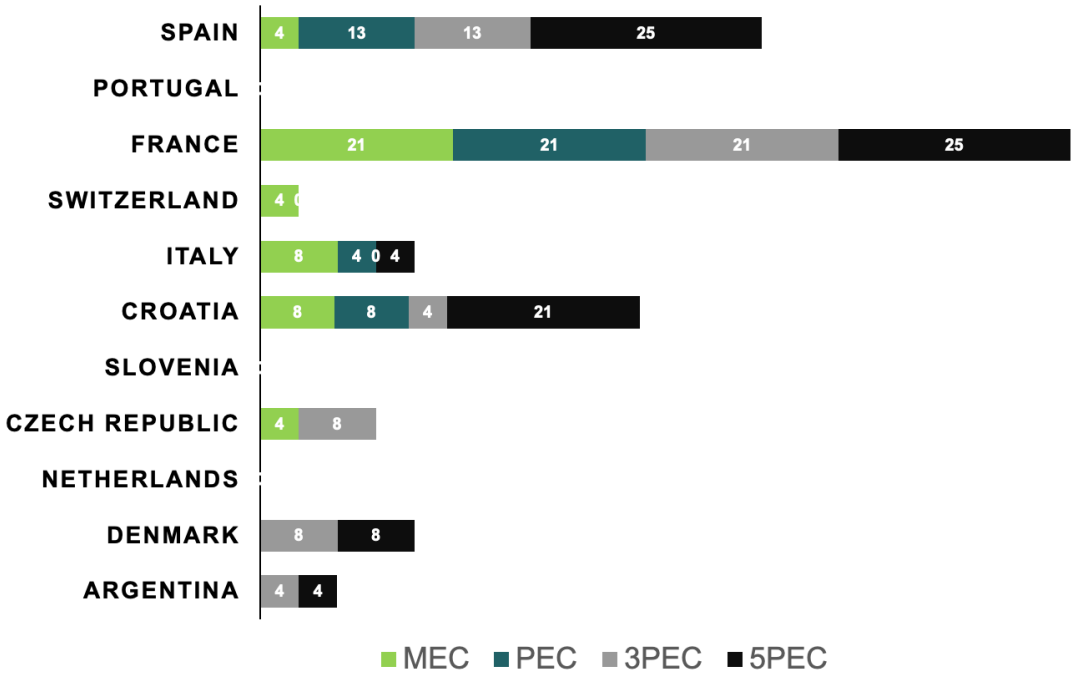

Figure 7.2 Percentage of immobilization of *Danio rerio* when exposed to distinct mixtures. MEC – measured environmental concentration; PEC – predicted environmental concentration.

ChE activity is depicted in Figure 7.3. ChE activity significantly decreased compared to the control (CTR) for Portugal, Switzerland, Slovenia under all tested conditions, except for Portugal. Additionally, a similar decrease in ChE activity was observed for The Netherlands after exposure to PEC and 3PEC, Spain at 3PEC, Croatia at 5PEC, and Italy and the Czech Republic at 3PEC and 5PEC. No effects on ChE activity were noted for the other France, Denmark and Argentina. Overall, a significant impact on ChE activity was evident for most of the mixtures tested, highlighting the toxicity of these pesticide mixtures on a key organism in aquatic systems. This reduction in ChE activity could have important consequences for the movement and behaviour of these fish in polluted aquatic environments.

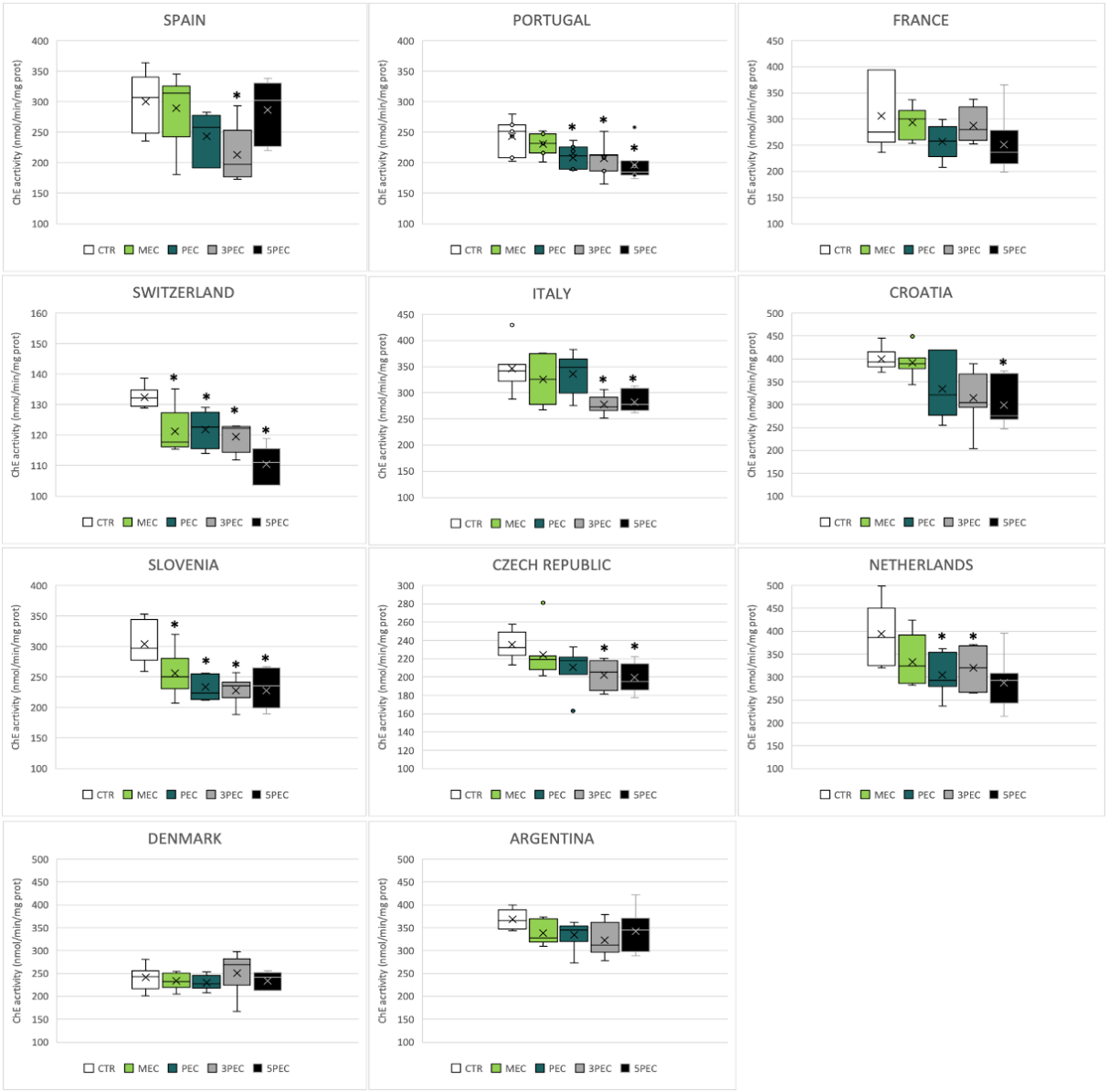

Figure 7.3 ChE activity (nmol/min/mg prot) of *D. rerio larvae* after CTR, MEC, PEC, 3PEC and 5PEC exposures for 96h. Upper edge of the box, line within the box and lower edge of the box, represents the 75th, 50th, and 25th percentiles. Vertical lines extend from the minimum to the maximum value, excluding outliers (circles) values. The significant differences were marked by (* $p<0.05$) respect to control.

To complement these results, the behavioral response was evaluated by analyzing various endpoints. Specifically, the total swimming distance (Figure 7.4) and the percentage of

distance in the outer zone (Figure 7.5) were measured to assess locomotor activity and swimming patterns, respectively.

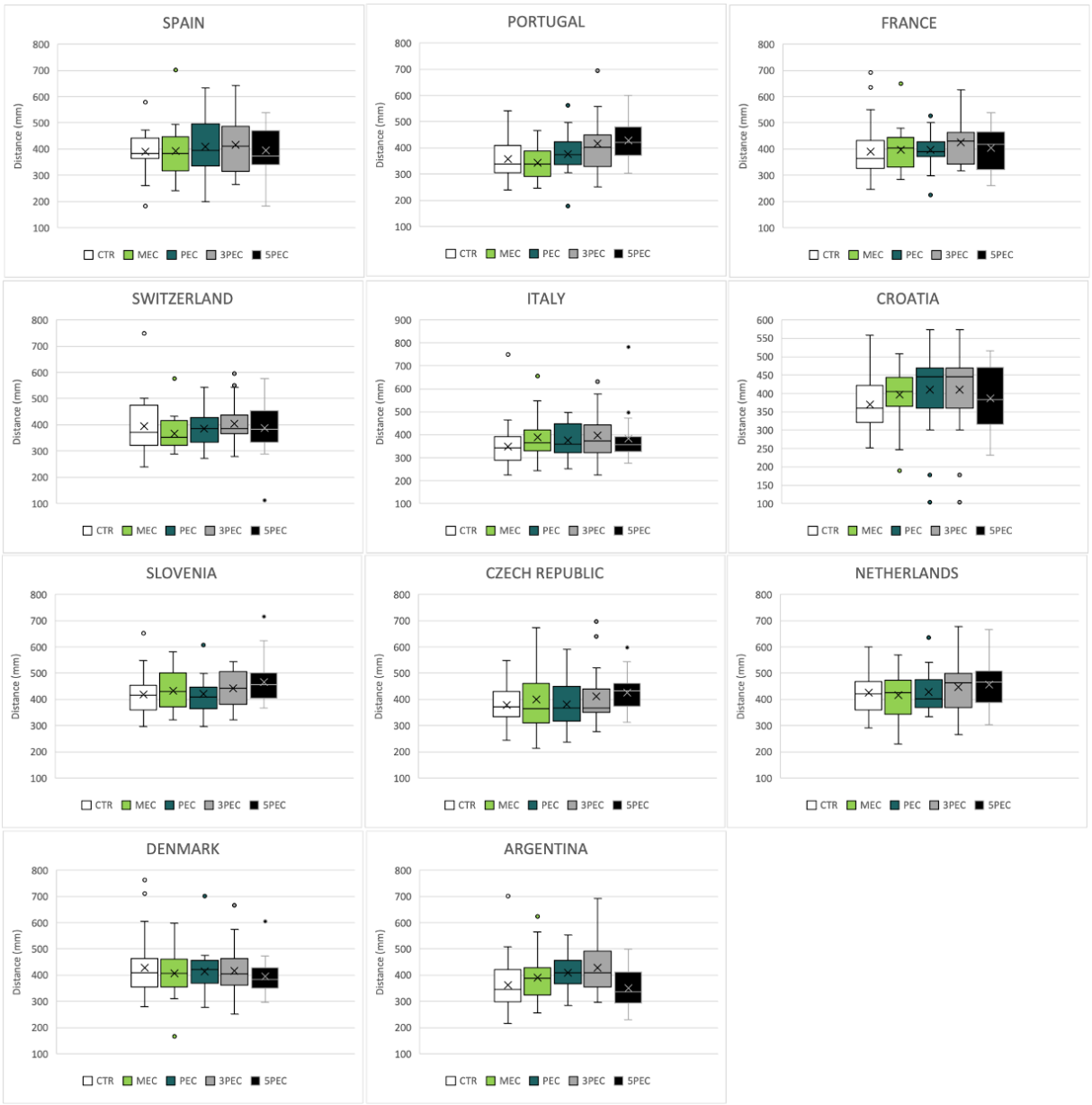

Figure 7.4 Total swimming distance as distance travelled in millimeters by *D. rerio* larvae after CTR, MEC, PEC, 3PEC and 5PEC exposures for 96h. Upper edge of the box, line within the box and lower edge of the box, represents the 75th, 50th, and 25th percentiles. Vertical lines extend from the minimum to the maximum value, excluding outliers (circles) values. The significant differences were marked by (* $p<0.05$) respect to control.

A low impact on locomotor activity was observed, with differences from the control (CTR) only noted for Portugal and the Czech Republic at 5PEC exposure, both showing increased swimming distances. These cases also corresponded to a significant impact on ChE activity.

Additionally, increased swimming distance was observed for Argentina at 3PEC, despite no detected effects on ChE activity. The low impact on locomotor activity, despite strong modulation of ChE activity, may be related to a possible oxidative stress response and other mechanisms that mitigate the potential impact on movement.

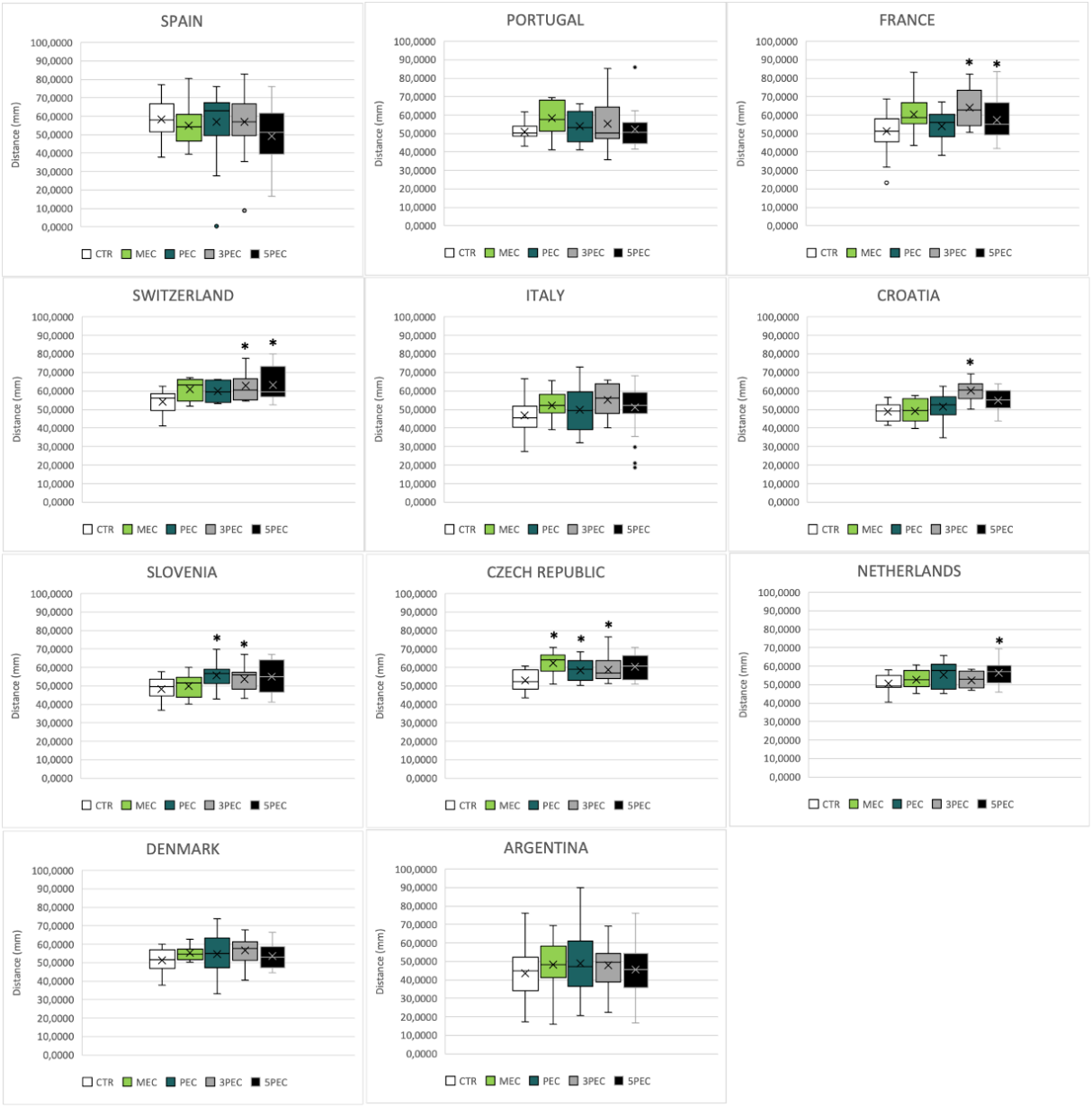

Figure 7.5 Swimming pattern expressed as percentage distance in the outer zone by *D. rerio* larvae after CTR, MEC, PEC, 3PEC and 5PEC exposures for 96h. Upper edge of the box, line within the box and lower edge of the box, represents the 75th, 50th, and 25th percentiles. Vertical lines extend from the minimum to the maximum value, excluding outliers (circles) values. The significant differences were marked by (* $p<0.05$) respect to control.

Additionally, the swimming pattern was analyzed by observing the percentage of distance in the outer zone, a parameter related to the escape capacity of the organisms. Under unfavourable conditions, larvae are expected to spend more time in the outer zone. This tendency was observed in France (MEC, 3PEC), Switzerland (3PEC, 5PEC), Slovenia (PEC, 3PEC), the Czech Republic (MEC, PEC, 3PEC), and for Croatia and The Netherlands at 5PEC exposure. Except for France, this stressful pattern was previously confirmed by decreased ChE activity and increased swimming distance in the case of the Czech Republic. These results are noteworthy because they reflect the impact of mixtures even at the realistic measured concentrations (MEC) as well as the predicted ones (PEC). Furthermore, it is crucial to underscore that these exposures are acute (96 hours), highlighting the need to investigate the potential long-term impact of these mixtures already present in aquatic systems.

7.4 Conclusions

- The zebrafish acute toxicity test showed only slight toxicity, with Croatia, France, and Spain having the highest toxicity levels, with a maximum immobilization of 25% at 5PEC.
- ChE activity significantly decreased compared to the control for Portugal, Switzerland and Slovenia, under most conditions, indicating a strong impact from the pesticide mixtures.
- Behavioural responses, including total swimming distance and the percentage of distance in the outer zone, were measured to assess locomotor activity and swimming patterns, revealing a low impact overall.
- Increased swimming distance, indicating a potential oxidative stress response, was observed for Portugal, the Czech Republic at 5PEC, and Argentina at 3PEC, despite no detected effects on ChE activity in the latter.
- The tendency of larvae to spend more time in the outer zone under unfavourable conditions was observed in multiple countries, correlating with decreased ChE activity and increased swimming distance, especially in the Czech Republic.

8 Acute immobilization test with *Daphnia longispina* (Native species)

8.1 Introduction

Daphnia longispina, a planktonic crustacean native to Eurasia, inhabits a wide range of stagnant freshwater bodies, from small ephemeral rock pools to large lakes. Unlike *D. magna*, which is primarily used in laboratory settings, *D. longispina* is a field native organism, providing a more realistic response when exposed to pesticide mixtures.

To study the acute effects of pesticide mixtures, organisms less than 24 hours old should be used. To minimize variability in results, it is recommended that the daphnids are not from the first or second brood progeny. Neonates must come from a healthy, well-established culture, showing no signs of stress such as high mortality, presence of males and ephippia, delayed production of the first brood, or discoloured animals. Test conditions, including light, temperature, and medium, should match culture conditions. The test follows the OECD 202 (2004) guidelines.

8.2 Methodology

The percentage of immobilized daphnids neonates (<24h) were evaluated after 48 hours of exposure to the pesticide's mixtures, at 18-22^{0}C, in the dark and with no food. Twenty animals, divided into four groups of five animals each were used at each test concentration (MEC, PEC, 3PEC and 5PEC) and controls (ASTM medium) (Figure 8.1).

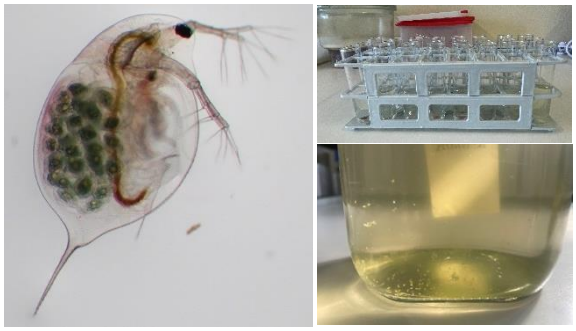

Figure 8.1 Cultures of *Daphnia longispina* and test tubes used to carry out the assays.

8.3 Results and Discussion

The Spain and France pesticide mixtures showed higher immobilization values for the PEC, 3PEC and 5PEC exposure (95/100%, 100% and 100%, respectively) (Figure 8.2). Those responses are like to the obtained with *D. magna* in an acute immobilization bioassay. However, when exposed to Switzerland, and Slovenia pesticide mixtures *D. longispina* recorded lower immobilization values for the PEC, 3PEC and 5PEC exposure than *D. magna*. Furthermore, Denmark, Netherlands, and Italy pesticide mixture also revealed a higher immobilization after 3PEC and 5PEC exposure (100%). Contrary, no effects were observed for Argentina, the Czech Republic, Switzerland and Portugal. The results obtained for Argentina and Portugal match with those obtained for *D. magna* in the acute immobilization test. The exposure to the MEC mixture did not cause *D. longispina* immobilization response in any mixture tested.

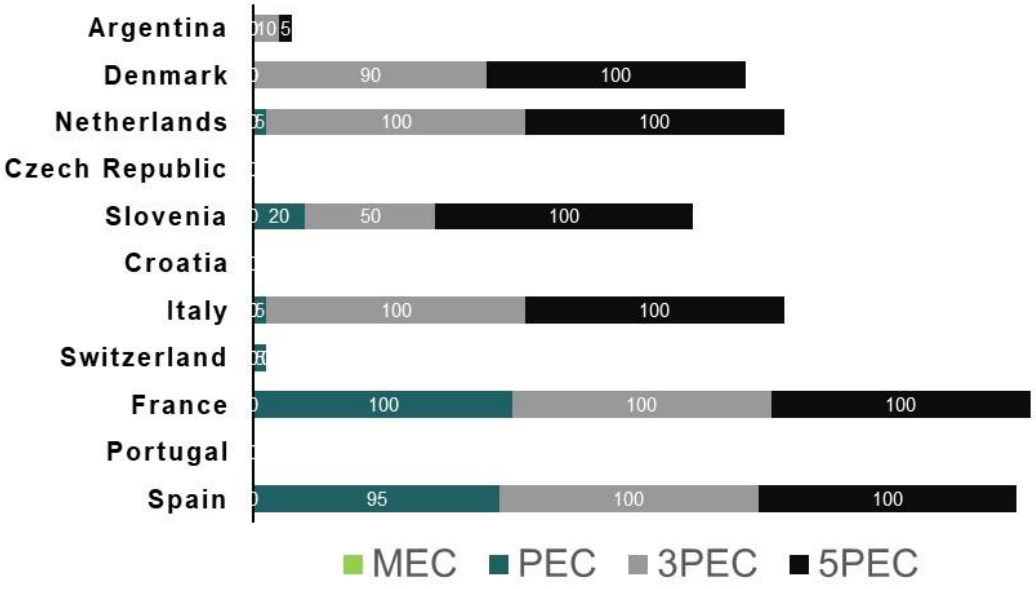

Figure 8.2 Percentage of immobilization of *Daphnia longispina* when exposed to distinct mixtures. MEC – measured environmental concentration; PEC – predicted environmental concentration.

8.4 Conclusions

- Pesticide mixtures from Portugal, Switzerland, Croatia, the Czech Republic, and Argentina did not affect the immobilization percentage of *D. longispina*.
- Pesticide mixtures from Spain and France cause the most significant negative effects, with a clear response based on the PEC concentration.

- Pesticide mixtures from Italy, Slovenia, The Netherlands, and Denmark showed a response starting at the 3PEC concentration, with values ranging from 50% to 100% for this concentration.

9 Reproduction test with *Daphnia longispina* (Native species)

9.1 Introduction

The reproduction test with *D. longispina* was adapted from the OECD 211 (2012) guideline established for *D. magna*. The primary objective of this test is to assess the effects of pesticide mixtures on the reproductive output of *Daphnia longispina*.

9.2 Methodology

Young female *D. longispina* (the parent organisms with less than 24hours at the start of the test), are exposed to a range of pesticides mixtures concentrations, namely MEC (Measured Environmental Concentration), PEC (Predictable Environmental Concentration), 3PEC and 5PEC and the control (ASTM Medium). For 21 days the total number of living offspring produced is assessed. Reproductive output of the parent animals can be expressed in other ways (e.g. number of living offspring produced per animal per day from the first day offspring were observed) but these should be reported in addition to the total number of living offspring produced at the end of the test.

In cases of parental mortality among exposed replicates, it is essential to determine whether the mortality follows a concentration-response relationship. If no such pattern is observed, replicates with parental mortality should be excluded from the analysis. Conversely, if a concentration-response relationship is present, the mortality should be attributed to the pesticide mixtures, and those replicates should be included in the analysis. If a parent animal dies due to mishandling, an unrelated accident, or is identified as male, the corresponding replicate should be excluded from the analysis. The survival of the parent animals and the time to production of the first brood should also be reported. Additional parameters, such as growth and possibly the intrinsic rate of population increase, can also be determined as endpoints.

Results of the acute immobilization toxicity test performed with *D. longispina* were useful in selecting an appropriate range of pesticides mixtures concentrations in the reproduction tests. Pesticides mixtures concentrations with LC_{50} (concentration of a substance that is

expected to cause death in 50% of a defined population of test organisms within a specified period) greater than 50% were excluded from chronic reproduction tests.

At the start of the test, the *D. longispina* newborns should be less than 24 hours old and not be from first or second brood progeny. They should be derived from a healthy culture and maintained in culture conditions (light, temperature, medium, feeding and animals per unit volume) similar to those to be used in the test.

The culture medium used in the reproduction test was ASTM. This medium also served as the control and as the dilution medium for the various concentrations of pesticide mixtures.

Parent animals are maintained individually in test vessels, each containing 10 mL of control medium or different pesticide mixtures for the first 8 days. After the first offspring, the volume used was 50 mL to avoid overpopulation and food limitations when the different broods are born. At least 10 animals are individually maintained at each pesticide concentration, and 12 animals in the control group. The test was conducted at 18-22°C in darkness to prevent light pesticide degradation. The test vessels remained unaerated throughout the duration of the test.

Feeding was done at least three times per week, coinciding with media changes. During the test, the parent animals were fed with living *Raphidocelis subcapitata* algal cells and to avoid dilution of exposure concentrations, well-concentrated algae suspensions were used.

Offspring produced by each parent animal were removed and counted daily from the appearance of the first brood to prevent the consuming food intended for the parent. Any aborted eggs or dead offspring were recorded. Mortality among the parent animals was also recorded daily, or at least as frequently as the offspring counts.

Oxygen concentration, temperature, hardness and pH values were measured at least once a week, in fresh and old media, in the control(s) and in the highest test substance concentration. During the test, the concentrations of the pesticides in the mixtures were determined at least one time.

9.3 Results and Discussion

The concentrations of the pesticide mixture selected for each country and used in *D. longispina* chronic reproduction test were based on the results of acute bioassays with the same species. All concentrations that resulted in more than 50% survival in the acute tests were included. The results obtained in these tests are represented in the Figure 9.1.

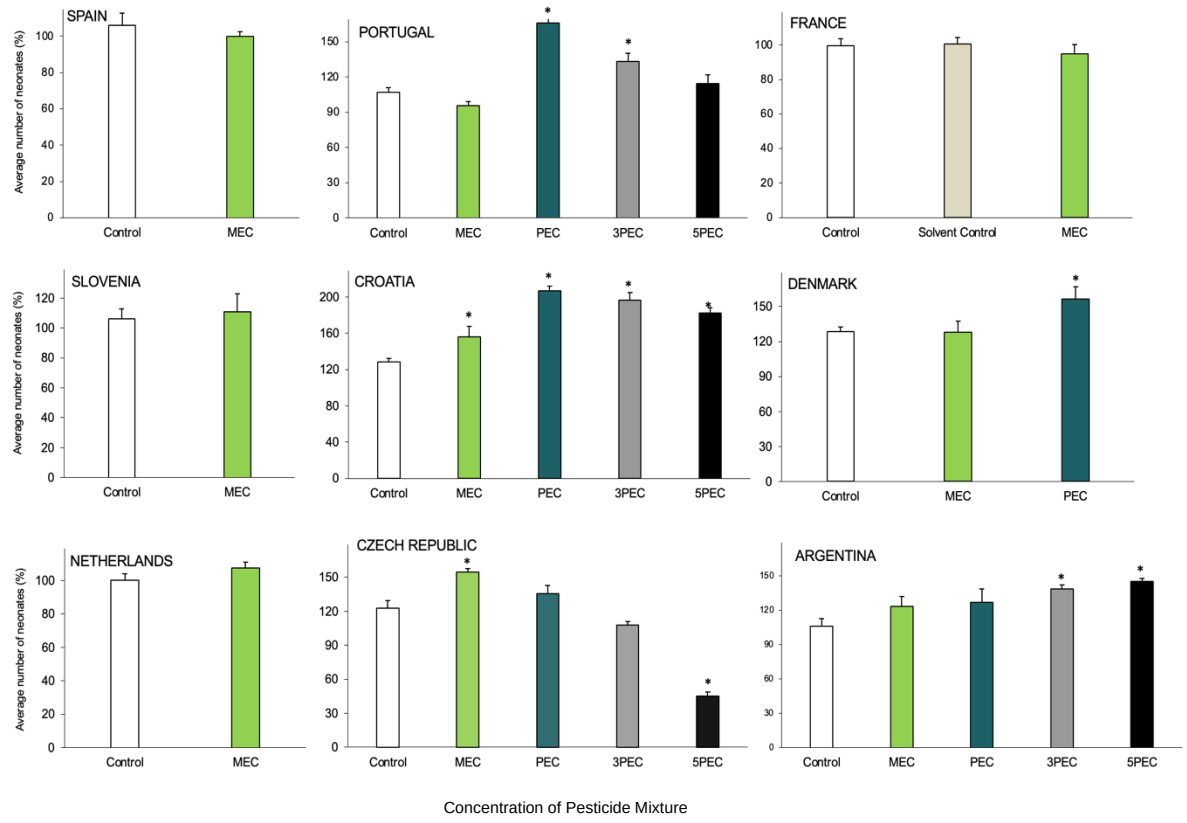

Figure 9.1 Percentage of the average number of *Daphnia longispina* neonates born during 21 days of exposure up to the 5ª offspring when exposed to distinct mixtures ± standard error. MEC – measured environmental concentration; PEC – predicted environmental concentration. The significant differences between treatments and control were marked by (*) $p<0.05$.

In the case of Portugal, Croatia, the Czech Republic and Argentina, four concentrations of pesticide mixture (MEC, PEC, 3PEC, and 5PEC) and a control were used. In contrast, for Spain, France, Slovenia, and The Netherlands, only one concentration (MEC) and a control were employed. A solvent control was included when necessary. For Denmark, two concentrations (MEC and PEC) were used in addition to a control.

For Portugal, the average number of neonates obtained after 21 days in 5 offsprings at MEC concentration was similar to the control. In PEC and 3PEC there was a stimulus with a significant increase in the number of newborns. However, there was a decrease in PEC to 5 PEC values. These values are in accordance with the average number of newborns in the 1st offspring, which in the PEC was 7,2 ±0,81 while in the control the value was 4,17 ±0,27. The average age of the 1st offspring had a value similar to the control (7,6± 0,15 days) while in 5PEC it decreases to 7,1 ±0,10 days. Regarding the number of offsprings in 21 days, the lowest value was in PEC concentration (4,3±0,42 offspring).

The number of newborns in the five offsprings over 21 days for Argentina, increased from MEC to 5PEC, with significant differences observed between 3PEC, 5PEC, and the control. The number of newborns in the first offspring aligned with the average number of newborns across all five offsprings. However, at the 3PEC concentration, the average age of the first offspring and the average number of offsprings decreased, and this concentration also exhibits higher mortality rates.

For Croatia, the stimulation of the average number of newborns across five offspring was similar to the one obtained for Portugal pesticide mixture, although there are statistical differences between all concentrations and the control. When comparing pesticides from these two countries, only the herbicides terbuthylazine and glyphosate were common. The Croatia pesticide mixture uniquely included the fungicide trifloxystrobin metabolite CGA and AMPA. The number of newborns in the first offspring decreased from control to 5PEC, while the number of days required to produce the first offspring increased.

As for the Czech Republic there was a significant positive stimulus in the MEC concentration, beginning to notice a decrease that is significant for the 5PEC concentration. The mixture of the Czech Republic pesticides had tebuconozole and terbuthylazine similar to Portugal, but with the exclusive fungicide Fenhexamid, which can help justify these responses.

For Spain, France, Slovenia, and The Netherlands, only the MEC and control concentrations were tested, and no significant effects were observed at these concentrations. For France, a solvent control was also used, which did not cause any effects at the tested concentrations.

For Denmark, the MEC concentration resulted in average newborn values similar to the control, whereas the PEC concentration showed a significant increase. Denmark's pesticide

mixture included the herbicide glyphosate, which was also present in the pesticide mixtures of Portugal, Croatia, and Slovenia, causing similar effects. Additionally, the fungicides fluopyram, Metalaxyl Metabolite CGA, and dimethomorph, which were unique to this mixture, may also contribute to the observed increase.

The pesticide mixtures from Italy and Switzerland had not yet been tested.

9.4 Conclusions

- For pesticide mixtures from Spain, Portugal, France, Slovenia, The Netherlands, Denmark and Argentina, the MEC concentration did not show significant differences compared to the control.
- However, for Croatia, the Czech Republic and Denmark mixtures, the MEC concentration was significantly higher than the control, resulting in an increased number of neonates in the first five offsprings.
- For Portugal and Croatia, pesticide mixtures stimulated the number of newborns in the first five litters at PEC, 3PEC, and 5PEC concentrations. However, in Portugal, Croatia, and the Czech Republic, the stimulation decreased from PEC to 5PEC.
  In contrast, the Argentina pesticide mixture showed an increase in the number of newborns after 21 days at the same concentrations.

10 Final considerations

10.1 Summary of the findings

The extensive ecotoxicological assessment conducted under the SPRINT project reveals that all tested aquatic species were impaired by pesticide mixtures, although the extent of the effects was highly dependent on the specific mixture and its composition. The findings underscore the importance of considering the combined effects of multiple pesticides, as real-world scenarios often involve exposure to such mixtures rather than individual chemicals.

Microalgae (*Raphidocelis subcapitata*):

- A moderate growth inhibition was observed at PEC, 3PEC, and 5PEC concentrations for pesticide mixtures from Portugal, Italy, Croatia, and the Czech Republic, with inhibition exceeding 30%. Mixtures from Spain, France, Denmark, and Argentina caused only slight growth inhibition. At MEC concentrations, none of the pesticide mixtures resulted in significant growth rate inhibition.

Macrophyte (*Lemna minor*):

- Expressive growth inhibition occurred with pesticide mixtures from Portugal, Switzerland, Italy, Croatia, Slovenia, the Czech Republic, the Netherlands, and Argentina. In contrast, mixtures from France, and Denmark resulted in minimal growth inhibition, suggesting lower toxicity. No toxicity was observed in Spain at any concentration. MEC concentrations had only a slight impact on growth, with the highest inhibition recorded in Argentina and the Czech Republic (13%).

Crustaceans (*Daphnia magna*):

- Significant negative effects were observed with mixtures from Spain, France, the Czech Republic, Slovenia, and the Netherlands, suggesting higher toxicity levels. In opposite, mixtures from Portugal, Denmark, and Argentina did not affect immobilization rates, indicating low acute toxicity. In Spain, while none of the pesticide concentrations caused growth inhibition in *L. minor*, severe immobilization effects were observed for *D. magna*.

Aquatic Insects (*Chironomus riparius*):

- Most mixtures, except for those from the Czech Republic, caused significant immobilization. High toxicity was particularly evident in mixtures from Argentina, Italy, and France, with total immobilization observed at certain concentrations, highlighting the sensitivity of this group to pesticide mixtures.

Fish Embryos (*Danio rerio*):

- Overall, the fish embryo test showed only slight toxicity, but the highest levels were found in mixtures from Croatia, France, and Spain. Significant effects on swimming behaviour and ChE activity were recorded, suggesting potential oxidative stress

responses and subtle sub-lethal effects with possible long-term implications for fish populations.

Native Crustacean (*Daphnia longispina*):

- **Acute Test:** Mixtures from Portugal, Switzerland, Croatia, the Czech Republic, and Argentina did not significantly affect immobilization rates. In contrast, significant negative effects were observed with mixtures from Spain, France, Italy, Slovenia, the Netherlands, and Denmark. These findings indicate that native species, potentially more representative of real-world scenarios, are also impacted by pesticide exposure in specific regions.
- **Reproduction Test:** Mixtures from Spain, Portugal, France, Slovenia, The Netherlands, Denmark, and Argentina did not significantly impact reproduction at MEC concentrations. However, significant increases in reproductive output were noted with mixtures from Croatia, the Czech Republic, and Denmark, especially at higher concentrations. This suggests that pesticide exposure may lead to complex reproductive responses, including potential hormesis or overstimulation under certain conditions.

10.2 Implications for policy and regulation

The findings from this comprehensive study highlight the urgent need for a paradigm shift in pesticide risk assessment and regulation. Current regulatory frameworks, which often focus on individual pesticides, may underestimate the real-world risks posed by complex pesticide mixtures. The significant regional differences observed in the study further underscore the necessity for localized risk assessments and tailored mitigation strategies.

To better protect aquatic ecosystems, it is recommended that regulatory bodies, such as the European Food Safety Authority (EFSA), incorporate whole-mixture approaches and consider cumulative and synergistic effects of pesticide mixtures in their risk assessment protocols. Additionally, implementing stricter controls and monitoring programs for pesticide use in agriculture, particularly in regions identified as having higher toxicity levels, could mitigate the adverse effects on aquatic life.

10.3 Future outlook

To better understand the risks of pesticides in aquatic ecosystems, it is essential to advance significantly through the integration of advanced approaches, a focus on multispecies and ecosystem-level assessments, improved modelling techniques, holistic tools to risk assessment, enhanced regulatory frameworks, and global collaboration.

Future risk assessments approaches will increasingly incorporate multispecies and ecosystem-level assessments, moving beyond traditional single-species tests that often fail to capture the complexities of real-world environments where multiple species interact. Multispecies assays and mesocosm studies, which simulate natural ecosystems, will provide even more ecologically relevant data. These methods will reflect the intricate interdependencies and potential cascading effects of pesticide exposure in aquatic ecosystems, offering a more comprehensive understanding of ecological impacts.

Advancements in ecological modelling and simulation techniques, such as the FOCUS (Forum for Co-ordination of pesticide fate models and their Use) simulation model, will enhance the predictive capabilities of environmental risk assessment by integrating diverse data sources to more accurately predict pesticide fate and transport.

Advanced technologies like high-throughput screening, genomics, and bioinformatics will enable precise identification of the molecular and biochemical effects of pesticides on aquatic organisms. Big data analytics and machine learning algorithms will facilitate the analysis of complex datasets, providing deeper insights into the interactions and cumulative impacts of multiple stressors on aquatic ecosystems.

Holistic approaches that consider multiple stressors, including chemical mixtures and environmental factors, will become more prevalent. Current regulatory frameworks often assess the toxicity of individual active ingredients in isolation, which may not reflect real-world scenarios. It will be adopted a more integrative approach, evaluating the combined effects of pesticide mixtures and their interactions with other environmental stressors, such as climate change and habitat degradation. Regulatory frameworks and policies will evolve to incorporate the latest scientific advancements and address emerging environmental challenges, shifting towards more dynamic and adaptive regulatory processes that can respond to new data and changing environmental conditions. Enhanced collaboration between regulatory agencies, researchers, and stakeholders will be crucial in developing and implementing effective risk management strategies.

The risk assessment of pesticides will place greater emphasis on understanding the linkages between ecological health and human health. Pesticide contamination in aquatic ecosystems can have far-reaching implications for human well-being, particularly through the contamination of drinking water sources and the bioaccumulation of toxic substances in the food chain. So, it will address these interconnected risks, promoting sustainable agricultural practices that protect both environmental and human health.

Global collaboration and data sharing will be crucial in advancing the understanding of pesticide risks. International initiatives to standardize risk assessment methodologies and exchange data across borders will improve the robustness and comparability of these studies.

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