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# Ecotoxicological assessment of PPPs' mixtures on trophic transfer and bio-amplification of PPPs for both terrestrial and aquatic compartments

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# 1 Introduction

Agriculture relies heavily on the application of Plant Protection Products (PPPs), such as herbicides, insecticides, and fungicides, to safeguard crops from pests and diseases, thereby boosting crop productivity. Common agricultural practices often involve using multiple PPPs, resulting in complex pesticide mixtures that enter the environment. Furthermore, farmers typically apply pesticides multiple times per year, increasing the range and concentration of pesticide combinations within agricultural ecosystems (Navarro et al., 2024; Silva et al., 2019; Weisner et al., 2021). Due to frequent and cumulative applications, pesticides can persist in the environment based on their individual half-lives, leading to the accumulation of residues in soil and water. This widespread presence of PPPs contributes to contamination in both terrestrial and aquatic ecosystems, posing potential risks to non-target species (Navarro et al., 2024; Panico et al., 2022; Ralf B. Schafer et al., 2011; Rico-Martínez et al., 2022).

Predicting the ecotoxicological effects of these pesticide mixtures is challenging due to their complex interactions, which may vary from additive to synergistic or antagonistic effects among different residues (Deneer, 2000; Weisner et al., 2021). Such interactions can amplify toxicity, potentially leading to harmful impacts on organisms even at concentrations deemed safe for single pesticide exposures (Panico et al., 2022). Consequently, the cumulative impacts of pesticide mixtures on terrestrial and aquatic organisms are often overlooked. Current regulatory risk assessments (Hardy et al., 2018) may underestimate the ecological risks posed by these mixtures, as they typically focus on the toxicity of individual active ingredients, failing to capture the full scope of potential risks associated with PPP mixtures.

As part of the objectives of WP4 in the SPRINT project, studies on the bioaccumulation of pesticide mixtures in organisms from the terrestrial and aquatic compartments were planned. Microcosm experiments were performed using different trophic levels, with a final aim to assess the trophic transfer of the pesticides and their potential for biomagnification.

The results from the ecotoxicological tests, obtained in WP4 and published in Deliverables 4.1 and 4.2, showed that the exposure to some mixtures was toxic

at environmentally relevant concentrations (e.g., the predicted environmental concentration PEC). The ecotoxicological assessments of these selected mixtures were conducted also using multi-species microcosms.

Microcosms are test units designed to increase realism compared to standardized laboratory experiments and therefore can be used as a higher-tier assessment. The concept of a microcosm experiment is to simulate a more natural scenario by, for example, including different trophic levels exposed to prolonged test periods. Although microcosms are less realistic than semi-field and field studies, they provide a more controlled environment. As a result, the common issues in semi-field and field studies, such as low reproducibility and high variability, are reduced (Burrows and Edwards, 2002). For these reasons, multi-species microcosms have been proposed as an intermediate tier between tier I (single-species) and higher-tier experiments (field studies) for pesticide risk assessment (Ernst et al., 2024).

In WP4, the microcosm experiments were part of the "new SPRINT indicators". These tests were planned to provide extra information on the effects of pesticide mixtures that are not covered by the low-tier standard single-species EFSA tests. For the terrestrial assessment, the bioaccumulation of a pesticide mixture in earthworms and terrestrial plants was measured in the microcosm tests, and a toxicokinetic model was applied (see section 2). For the aquatic assessment, bioconcentration, bioaccumulation, and bio-amplification through trophic transfer were evaluated using a microcosm that incorporated three distinct trophic levels, simulating a simplified food chain. This model included a producer (watercress: *Nasturtium officinale*), a primary consumer (freshwater shrimp: *Atyaephyra desmarestii*), and a secondary consumer (crayfish: *Procambarus clarkii*) (see section 3).

# 2 Terrestrial microcosm experiment

The main aim of the terrestrial microcosm is to assess the bioaccumulation of a realistic pesticide mixture in earthworms and plants. This experiment is a helpful tool to provide insights on the transfer of pesticides to the food chain via earthworms (ecosystem) or via edible plants (human food and animal feed).

Using a tiered approach, the pesticide mixtures were selected based on the results from the toxicity tests reported in D 4.1. For the terrestrial microcosm, the mixture selection was based on the results from the tests with earthworms. Earthworms were chosen as the model organism due to their importance to the soil compartment and for being highly used in the risk assessment of pesticides. In D4.1, it was observed that the mixture from the Czech Republic (CZ) was the most toxic for the single-species tests with 3 earthworm species, and therefore, chosen for the terrestrial microcosm experiment.

## 2.1 Methodology

### 2.1.1 Spiking procedure

The terrestrial microcosm experiment was based on previous work conducted at Masaryk University (Šudoma et al., 2019). A natural soil was collected from Unifarm, WU (Wageningen, The Netherlands). The soil was air-dried and shipped to Masaryk University. This soil was chosen due to its low content of organic carbon (1.6%), since the bioavailability of pesticides tends to increase in soils with low organic content. For each concentration, 12 kg of soil were pre-incubated (4 days, 50% of water holding capacity, dark, room temperature) to stimulate the microbial community, prior to the addition of the pesticides. Ten percent of soil (in dry weight) was spiked with pesticide stock solutions in acetone. Three concentrations of the pesticide mixture were used (Table 2.1). The first concentration tested was based on the medians of the measured concentrations (MEC) of the 5 selected pesticides. The second concentration was based on the highest predicted environmental concentrations (PEC) in soil after pesticide application retrieved

from EFSA documents. The third concentration was 5 times PEC value (5PEC). After the addition of the pesticide solutions, the soil was left to evaporate overnight. The next day, water loss was replenished, and the two portions of spiked and non-spiked soils were mixed. A negative and a solvent control were prepared by adding the same amount of water and/or acetone as in the treatments. Soil samples (5-10 g) were immediately stored at -20 °C after spiking for the later chemical analyses.

Table 2.1: Concentration of pesticides used in the terrestrial microcosm experiment.

| | µg/kg | | |
|---|---|---|---|
| | MEC | PEC | 5PEC |
| Azoxystrobin | 14 | 394 | 1970 |
| Boscalid | 18 | 396 | 1980 |
| Dimoxystrobin | 17 | 47 | 235 |
| Lambda-cyhalothrin | 2.4 | 33 | 164 |
| Thiophanate-methyl | 160 | 5533 | 27665 |

### 2.1.2 Test design

One kg of soil was added to each test pot, and 4 replicates were prepared for each concentration. In each pot, 10 adults *Lumbricus rubellus* with developed clitellum were weighted and added into the pots. The earthworms were obtained from The Bryophyta Nursery company (Cebazan, France) and acclimated in the test soil moistened to 50% water holding capacity and 7 mg cow manure per kg soil for 1 week before the experiment. Then 8 pre-germinated lettuce seeds (at 22 °C in the dark for 24 h) were planted in the soil with radicle down. The pots were incubated in controlled conditions in a greenhouse (Figure 2.2). The soil moisture content was adjusted 3-4 times a week, and 2 g dried cow manure were added to feed the earthworms every two weeks (14, 28, and 42 days). The cow manure was purchased as dry pellets and crushed into powder with a mixer.

Figure 2.2: Microcosm experiment on days 7 (top left), 28 days (top right), and 56 days (bottom).

After 14, 28, and 56 days, 4 pots of each treatment were sacrificed: 1) plants were removed, washed with water, and dried with towel paper, the roots and leaves were separated (Figure 2.3A), dried, weighted (dry weight), and frozen at -20 °C; 2) earthworms were collected from the soil, counted, washed, dried with towel paper, weighted (fresh weight), placed into a Petri dish with wet filter paper to depurate for 24 h (Figure 2.3B), and then frozen at -20 °C; 3) 30 g soil was frozen at -20 °C for pesticide analysis.

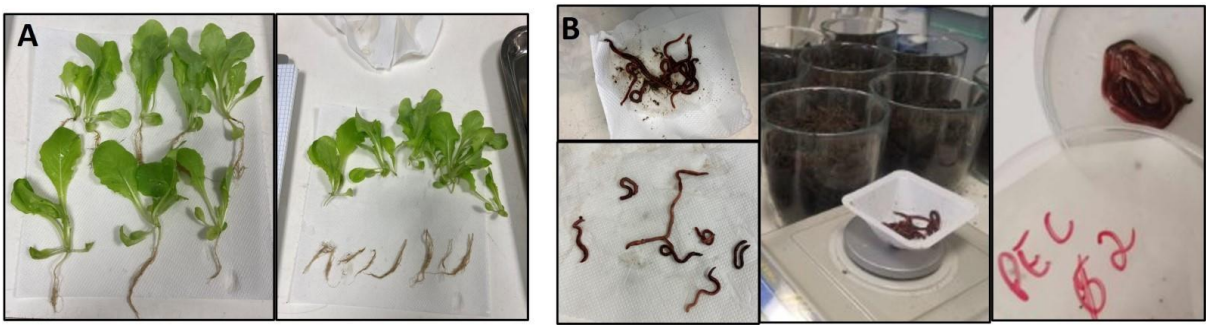

Figure 2.3: (A) Collection and treatment of lettuce samples. Plants were removed from the soil and leaf and root separated. (B) Earthworms were removed from the soil, weighted, and placed in Petri dishes for depuration.

### 2.1.3 Pesticide analysis

All samples were placed in 50 mL falcon tubes, lyophilized for 2 days, and had their weight recorded. For soil samples, a portion of 5-10 g of frozen stored soil was used. For earthworms, the surviving organisms of each replicate (pot) were pooled together. For the lettuce roots and leaves, due to the low sample amount, the biomass from all 4 replicates was pooled in one pseudo-replicate at 14 d, and in two pseudo-replicates at 28d. At 56d, the roots and leaves biomass were sufficient for the replicates to be analyzed separately. For pesticide analysis, the QuEChERS extraction method was used. Earthworms and lettuce samples were homogenized by adding a ceramic homogenizer in the tubes and shaking for 5 min (Geno/Grinder 2010). Samples were spiked with D6-metolachlor (used as surrogate standard) and mixed with 5 mL Millipore water and 10 mL acetonitrile. The plastic tubes were shaken 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. The tubes were then vortexed and centrifuged (5 min, 3500 rpm, -5 °C), and the supernatant was collected. For earthworms and lettuce, the supernatants were transferred to dispersive SPE kits to remove lipids and pigments, and centrifuged (5 min, 3500 rpm, -5 °C). For the LC-MS/MS analysis, 0.5 mL of the supernatant was transferred to 2 mL vials and diluted with water (1:1). For the GC-MS/MS analysis, 0.1 mL was transferred to 1 mL conical vials and 10 µL of PCB 162 was added.

The limit of quantification (LOQ) for LC-MS/MS (azoxystrobin, boscalid, dimoxystrobin, thiophanate-methyl) was 2, 16, 13, and 5 µg/kg for soil, earthworm, root, and leaf samples, respectively. The LOQ for GC-MS/MS (lambda-cyhalothrin) was 0.09, 0.01, 0.02, and 0.01 µg/kg for soil, earthworm, root, and leaf samples, respectively.

### 2.1.4 Uptake kinetic modeling

The change in pesticide levels in the soil during the experiment was modeled using a first-order kinetic equation:

$$C_{(t)} = C_0(e^{-k_0 t})$$

where $C_{(t)}$ is the concentration of the pesticides in the soil at time t, $C_0$ is the initial concentration of the pesticides in soil (µg/kg), $k_0$ is the degradation rate constant in soil ($day^{-1}$), and t is the time (day).

The uptake kinetics in earthworms was modeled using a first-order one-compartment model:

$$Q_{(t)} = \frac{k1}{k2 - k0} C_0 \, (e^{-k0t} - e^{-k2t})$$

where $Q_{(t)}$ is the internal concentration at time t ($\mu g/kg_{organism}$), $k_1$ is the uptake rate constant ($g_{soil}/g_{organism}/day$), $k_2$ is the elimination rate constant ($day^{-1}$), $k_0$ is the degradation rate constant ($day^{-1}$), $C_0$ is the initial concentration of the pesticides in soil (µg/kg), and t is the time (day).

The bioaccumulation factor (BAF) in earthworms was calculated as the ratio between $k_1$ and $k_2$. For lettuce, the bioconcentration factor (BCF) was calculated as the ratio between concentration and soil concentration at 56 days.

## 2.2 Results and Discussion

***Soil***

The soil concentrations measured at the beginning of the experiment were 52-84% for azoxystrobin, 80-113% for boscalid, 54-68% for dimoxystrobin, 100-128% for lambda-cyhalothrin, and 1-56% for thiophanate-methyl of their nominal concentrations in MEC, PEC, and 5PEC. The measured values were used in further calculations for the first-order kinetic model for degradation of the substances in soil (Figure 2.4). Azoxystrobin levels decreased faster at MEC, with 44% of the initial measured concentration at 14 days, and 35 and 32% at days 28 and 56. At PEC and 5PEC, azoxystrobin levels were 81-88% of the initial measured concentration after 14 days, and decreased to 58-60% after 56 days. Boscalid and dimoxystrobin had a fast degradation in the first 14 days, with 31-48% of initial

measured concentration across all concentrations. After 28 and 56 days, the degradation was slower and the concentrations were similar to those obtained at 14 days, being 30-39% of initial measured concentrations. Lambda-cyhalothrin showed a slow degradation after 14 and 28 days, with concentrations within 70% of the initial concentration after 28 days. After 56 days, the concentrations were 37-50% of the initial concentration. The concentration of thiophanate-methyl decreased levels below LOQ of 2 µg/kg after 14 days at MEC. At PEC and 5 PEC, the concentrations were 7-10% of the initial ones after 14 days, and gradually decreased to 1-4% after 56 days.

Considering the known DT50 for these substances in soil (78.0 for azoxystrobin, 484.4 for boscalid, 210.0 for dimoxystrobin, 175.0 for lambda cyhalothrin, and 0.5 days for thiophanate-methyl; PPDB), the only substance with such quick degradation can be thiophanate-methyl. For the other substances, the degradation is somehow in line with the DT50s.

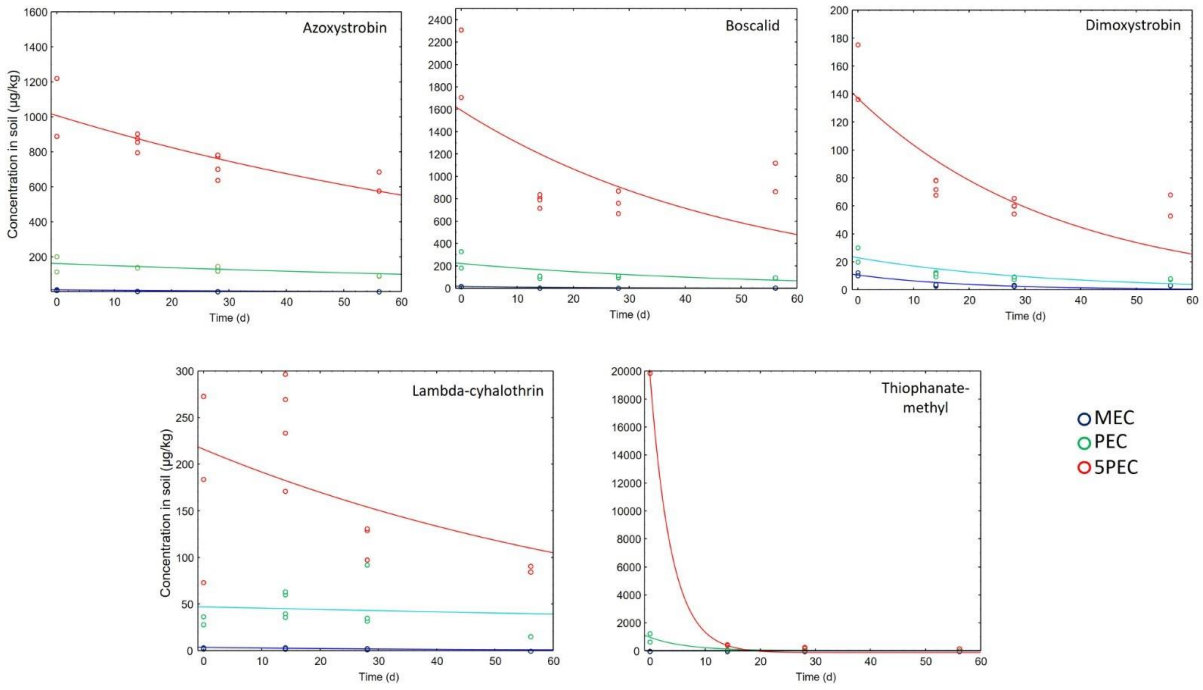


Figure 2.4: Soil concentration of the pesticides over time at median environmental concentration (MEC), predicted environmental concentration (PEC), and 5 times PEC (5PEC). Lines represent the first-order kinetic model.

### *Earthworms*

The results showed that the bioaccumulation in earthworms varied among pesticides and time points (Figure 2.5). At MEC exposure, all levels were below LOQ after 56 days. Moreover, it was not possible to measure the earthworms at 5PEC, due to the 100% mortality after 56 days of exposure.

Azoxystrobin had levels below LOQ at MEC exposure at all time points (azoxystrobin soil nominal concentration of 14 µg/kg). At PEC and 5PEC, azoxystrobin levels averaged 41 and 150 µg/kg, respectively, after 14 days. After 28 and 56 days, the concentrations in all samples were below LOQ. These results are in line with Bao et al. (2024) who observed that azoxystrobin concentrations in *Eisenia fetida*, exposed at 5 mg/kg, increased in the first 7 days of exposure, followed by a decrease from 14 to 28 days.

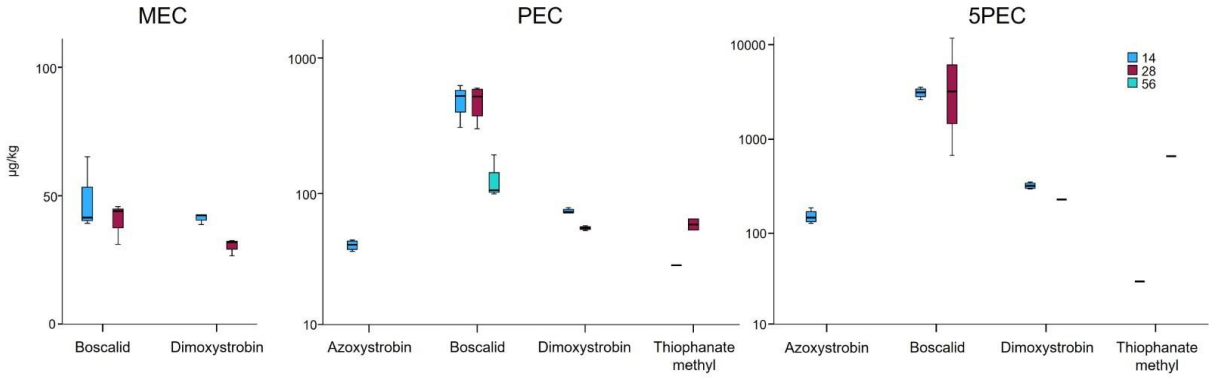


Figure 2.5: Boxplot of the concentration of pesticides in earthworms (*Lumbricus rubellus*) after 14, 28, and 56 days of exposure in measured environmental concentration (MEC), predicted environmental concentration (PEC), and 5 times PEC (5PEC). Box represents the interquartile range (quartile 1 - quartile 3), the whiskers represent maximum and minimum values, and the lines represent the median values. Note that the Y axis has different ranges for different concentration groups. The Y-axis of PEC and 5 PEC are shown in the log scale.

Boscalid concentrations in earthworms seemed to be rather stable between days 14 and 28 in all exposure concentrations. After 56 days, a decrease in boscalid concentration was observed at MEC (below LOQ) and PEC, indicating that the earthworms were able to eliminate boscalid only after 28 days. No study on boscalid accumulation in earthworms was found in the literature for comparison.

Dimoxystrobin concentration in earthworms decreased from 14 to 28 days at all exposure concentrations, until level <LOQ after 56 days at MEC and PEC. Dimoxystrobin has previously shown a high capacity for bioaccumulation in earthworms in a field experiment (Nélieu et al., 2020). Nevertheless, a decrease in earthworm body concentration was observed with time, which was related to a decrease in available residue in the soil.

For lambda-cyhalothrin, the concentrations in earthworms were <LOQ in all samples. In the literature, the level of lambda-cyhalothrin in the earthworm *E. fetida* exposed at 5 mg/kg peaked after 5 days and was approximately 750 µg/kg (Chang et al., 2016). However, the exposure concentration in Chang et al. (2016) was more than 30 times higher than our highest concentration. Therefore, it could be that lambda-cyhalothrin was not detected in our samples because of the low exposure concentrations, which ranged from 2.4 µg/kg in MEC to 164 µg/kg in 5PEC.

For thiophanate-methyl, the concentrations in earthworms increased from 14 to 28 days. These results were most likely influenced by the fact low number of samples where thiophanate-methyl was detected (i.e., one or two replicates for each time point). Therefore, the low number of replicates makes it difficult to ensure that its concentrations in earthworms indeed increased with time.

The uptake kinetics in earthworms and the parameters are displayed in Figure 2.6. The calculations were not performed for thiophanate-methyl and lambda-cyhalothrin due to the small number or no samples detected >LOQ. Azoxystrobin showed the highest elimination rate constant (k2) and the lowest uptake rate

constant (k1) of all 3 pesticides. Bao et al. (2024) observed that the decrease in earthworm body concentration after 14 days was related to the decline in azoxystrobin soil concentrations. Although a decline in soil concentration could explain the low k1, the k2 parameter is solely related to the detoxification ability of the organism. Boscalid and dimoxystrobin had higher k1 values and lower k2 in comparison to azoxystrobin. Still, the earthworms were able to eliminate and/or metabolize boscalid and dimoxystrobin if the exposure concentration is low enough. Their levels were below LOQ after 56 days at MEC, but not at PEC and 5PEC.

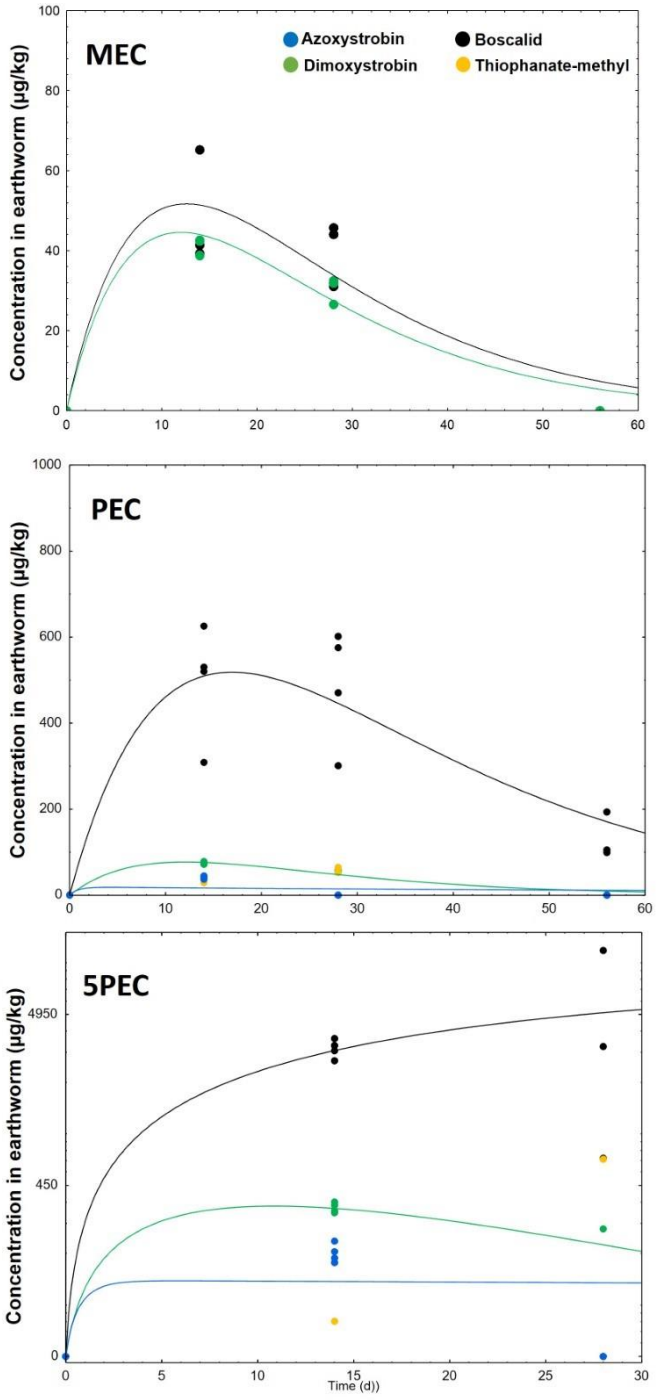


Figure 2.6: Toxicokinetics of azoxystrobin, boscalid, dimoxystrobin, and thiophanate-methyl in earthworms exposed to the median measured environmental concentration (MEC), predicted environmental concentration (PEC), and 5 times PEC (5PEC).

The bioaccumulation factors in earthworms, calculated as the ratio between k1 and k2, were 0.12 and 0.08 for azoxystrobin at PEC and 5PEC, respectively. For boscalid, BAF ranged from 2.0 to 7.3, and for dimoxystrobin, BAF ranged from 1.6 to 5.4.

***Lettuce***

The concentration of the pesticides in lettuce root and leaves can be seen in Figure 2.7. In root samples, boscalid was the only pesticide detected in all exposure concentrations, while azoxystrobin, dimoxystrobin, and lambda-cyhalothrin were detected at PEC and 5PEC. The fungicide thiophanate-methyl was not detected in any of the samples from both root and leaves. In leaf samples, no pesticide was detected at MEC. Boscalid was detected at PEC and 5PEC, and azoxystrobin and dimoxystrobin were detected at 5PEC. Unlike the root samples, lambda-cyhalothrin was not detected in leaf samples.

Boscalid had the highest uptake among the pesticides in both root and leaves, indicating that the translocation from root to leaf occurred at some level. Azoxystrobin and dimoxystrobin also showed potential for root-leaf translocation, however in lower levels than boscalid. Lambda-cyhalothrin was only detected in the root and at lower levels than the other pesticides, which can indicate that if translocation occurred it was not sufficient to be detected.

In most studies on bioaccumulation in terrestrial plants, pesticides are directly applied to the plants, based on the recommended application rates. In the present microcosm experiment, we aimed to understand the bioaccumulation of the pesticide residues in soil. Therefore, the plants were exposed via root uptake. These different routes of exposure can affect the outcomes. For example, (Wołejko et al., 2016) observed that azoxystrobin had a higher half-time of 4.7 days in comparison to boscalid (3.2 days) when plants were sprayed with both pesticides as formulation. These findings are not fully in agreement with our results, where boscalid bioaccumulation was higher than azoxystrobin. This may indicate that the

uptake and elimination of pesticides may differ depending on several factors, such as the exposure route.

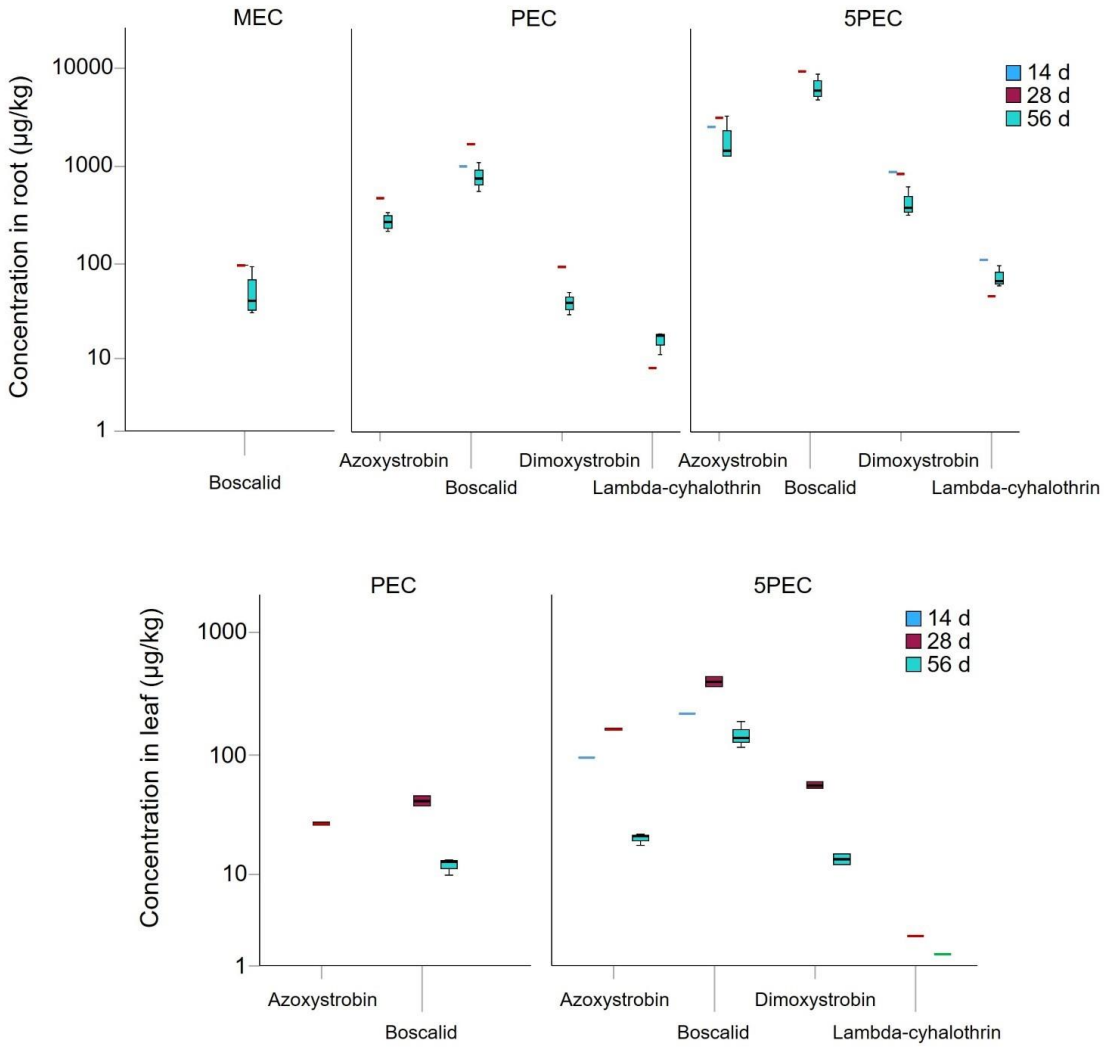


Figure 2.7: Boxplot of the concentrations of azoxystrobin, boscalid, dimoxystrobin, and lambda-cyhalothrin in lettuce (root on the top and leaf on the bottom) exposed to the median measured environmental concentration (MEC), predicted environmental concentration (PEC), and 5 times PEC (5PEC). Box represents the interquartile range (quartile 1 - quartile 3), the whiskers represent maximum and minimum values, and the lines represent the median values. The Y-axis is shown in the log scale.

The current maximum residue levels (MRL) of azoxystrobin, boscalid, and dimoxystrobin in lettuce are 10, 50, and 0.01 mg/kg (EC, 2024). For azoxystrobin and boscalid, the concentrations measured in lettuce leaves were all below the MRL. Dimoxystrobin concentrations exceeded the MRL (10 µg/kg) at 5 PEC exposure, where the concentration (average±SD) measured in the leaves was 57±5 µg/kg at 28 d. The concentration, however, decreased to 13±2 µg/kg after 56 days.

The BCF in roots and leaves calculated as the measured concentration in sample divided by the measured concentration in soil after 56 days are displayed in Table 2.2. In roots, the BCF were above 1 for all pesticides, except for lambda-cyhalothrin. In leaves, BCF was lower compared to roots, ranging from 0.01 to 0.2.

Table 2.2: Bioconcentration factor of the pesticides in lettuce root and leaves exposed to the median measured environmental concentration (MEC), predicted environmental concentration (PEC), and 5 times PEC (5PEC) after 56 days of exposure.

| | Root | | | Leaves | | |
|---|---|---|---|---|---|---|
| | MEC | PEC | 5PEC | MEC | PEC | 5PEC |
| Azoxystrobin | - | 3.0 | 3.0 | - | - | 0.03 |
| Boscalid | 7.5 | 8.4 | 6.5 | - | 0.1 | 0.1 |
| Dimoxystrobin | - | 5.0 | 7.1 | - | - | 0.2 |
| Lambda-cyhalothrin | - | 0.2 | 0.4 | - | - | 0.01 |

# 3 Aquatic microcosm experiment

A microcosm experiment combining 3 distinct trophic levels was implemented using a simplified trophic chain model, including a producer (watercress: *Nasturtium officinale)*, a primary consumer (shrimp: *Atyaephyra desmarestii*) and secondary consumer (red swamp crayfish: *Procambarus clarkii*). These species were selected due to their sufficient biomass for chemical analysis and their documented sensitivity to contaminants. These species were exposed to two pesticides mixture from Spain and The Czech Republic, two experimental conditions, CTR (APW (Artificial Pond Water) medium) and PEC, based on results obtained from previous monospecific ecotoxicological assays. Additionally, preliminary survival tests were conducted with the three organisms, varying exposure times to establish optimal conditions for trophic transfer studies. This approach aimed to balance the exposure conditions, considering the requirements of all three organisms simultaneously. The multi-species microcosm was used to evaluate the bioconcentration and bio-amplification of pesticides in each organism and to assess pesticides transfer from the producer to primary consumers, and from primary consumer to secondary consumers.

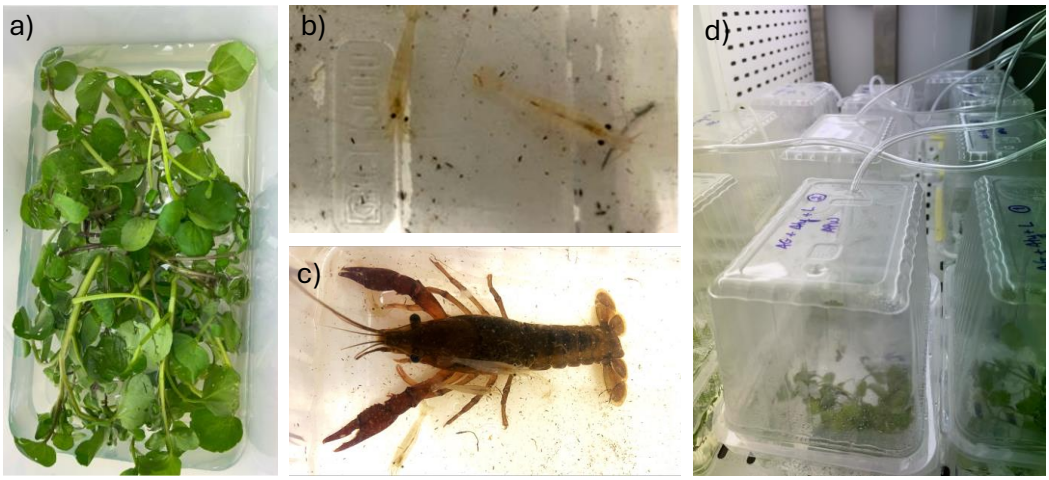


Figure 3.1: Species and apparatus used in the aquatic microcosm experiments. a): the producer (watercress, *Nasturtium officinale*); b) the primary consumer (shrimp, *Atyaephyra desmarestii*); c) the secondary consumer (red swamp crayfish, *Procambarus clarkii*); d) Vessels used to conduct multispecies experiments.

## 3.1 Tested pesticides mixtures

- The mixture selection for the aquatic microcosm was based on the results of monospecific toxicity tests conducted with various aquatic species (Deliverable 4.2). Since all 11 Case Study Sites (CSS) mixtures exhibited some level of toxicity, two CSS mixtures, from the Czech Republic (CZ) and Spain (SP), were chosen for the aquatic microcosm experiments. These two mixtures were selected based on their consistent toxicity across different aquatic organisms and their distinct chemical compositions. The Czech mixture contained one herbicide and four fungicides, while the Spanish mixture included two insecticides and three fungicides (Table 3.1). After the selection of the substances for each pesticide mixture, the microcosms experiments were performed using the Control (CTR) and the Predicted Environmental Concentration (PEC). While the PEC was confirmed through chemical analysis, all results are presented using nominal PEC. This approach ensures consistency and clarity in data presentation.

Table 3.1: Pesticide mixtures selected for microcosm experiments in Spain and the Czech Republic. Insecticides are represented in grey, fungicides in blue, and herbicides in green.

| Country | Pesticides | PEC (µg/l) |
|---|---|---|
| Spain (ES) | Chlorantraniliprole | 34.83 |
| | Fludioxonil | 14.20 |
| | Azoxystrobin | 110.8 |
| | Fluopicolide | 103.8 |
| | Acetamiprid | 4.970 |
| The Czech Republic (CZ) | Terbuthylazine | 243.5 |
| | Fluopicolide | 103.8 |
| | Fludioxonil | 14.20 |
| | fenhexamid | 312.0 |
| | Tebuconazole | 1.600 |

## 3.2 Preliminary growth inhibition test and survival tests

These preliminary tests assessed growth inhibition in *Nasturtium officinale* and survival rates in *Atyaephyra desmarestii* and *Procambarus clarkii* following exposure to pesticide mixture (PEC) concentrations from Spain and the Czech Republic. The goal was to identify optimal exposure conditions for future trophic transfer studies by using four durations (24, 48, 72, and 96 hours) under controlled conditions: temperature (19±2°C), light intensity (4440-6500 lux), photoperiod (16L:8D), no food, and aerated vessels.

### Growth Inhibition test with *Nasturtium officinale*

The growth inhibition test exposed *N. officinale* to the pesticide mixture (at PEC concentrations) and a control (CTR: APW medium) for up to 96 hours. Each assay vessel contained 250 ml of PEC or APW medium and approximately 12–15 g of *N. officinale*. Results showed that *N. officinale* continued to grow over the 96-hour period, with a biomass increase of 32% in the control and 16% in the PEC treatment (Figure 3.2).

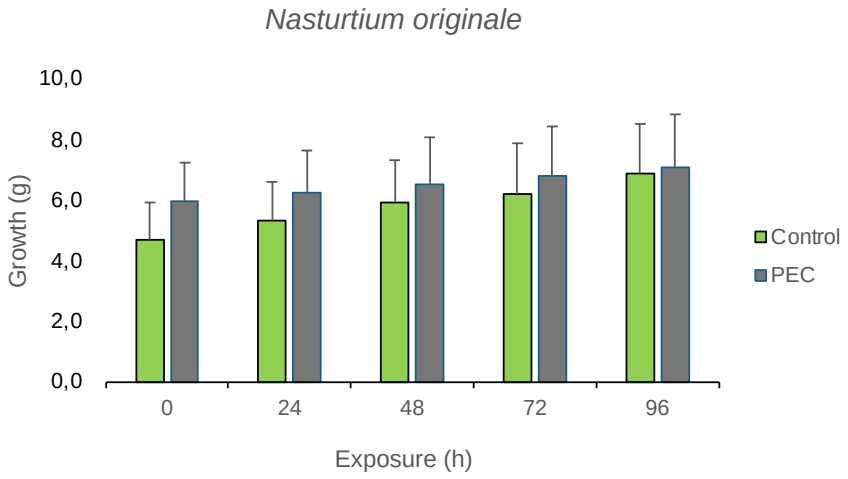


Figure 3.2: Average growth (g) ± standard deviation of *N. officinale* (watercress) exposed to the control (CTR) and pesticide mixture (PEC) over 96 hours.

### Survival test with *Atyaephyra desmarestii*

In the survival test, *A. desmarestii* was exposed to PEC and control conditions for the four specified durations (24, 48, 72, and 96 hours). Assays were conducted in 300 ml vessels, with five replicates per treatment and 10 organisms per vessel, and dead individuals were removed and counted every 24 hours. Results indicated a reduction in survival, with a 30% decrease in the control and a slightly higher reduction of 37% in the PEC treatment over 96 hours (Figure 3.3). This observed mortality likely reflects the organisms' adjustment from field to laboratory conditions rather than the toxicity of the pesticides. While mortality did not reach the critical 50% threshold, the results suggest adjusting the initial number of organisms in future tests to account for this acclimation effect.

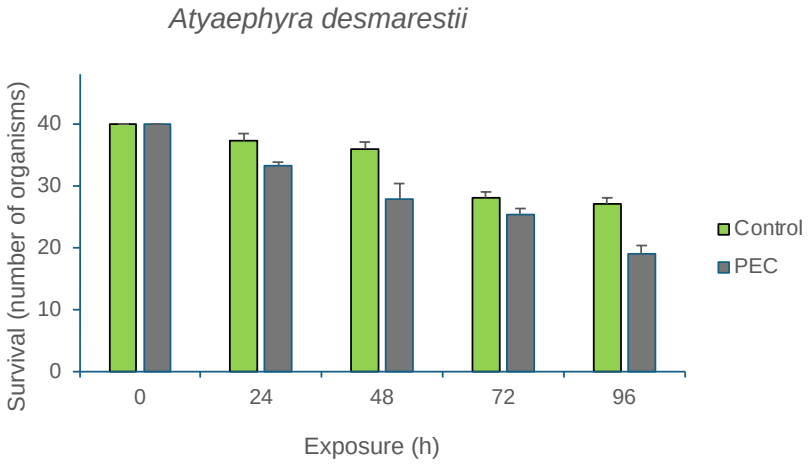


Figure 3.3: Average survival (number of live organisms) ± standard deviation of *A. desmarestii* (shrimp) exposed to the control (CTR) and pesticide mixture (PEC) over 96 hours.

### Survival test with *Procambarus clarkii*

The secondary consumer, *P. clarkii*, was exposed to PEC and control conditions under similar durations, in containers holding 500 ml of medium, with one crayfish per container and five replicates per treatment. The survival rates recorded at each exposure period revealed no significant mortality, with 97% survival in the control and 95% in the PEC treatment (Figure 3.4). These findings indicate that *P. clarkii* is suitable for 96-hour exposure studies without significant impacts on survival.

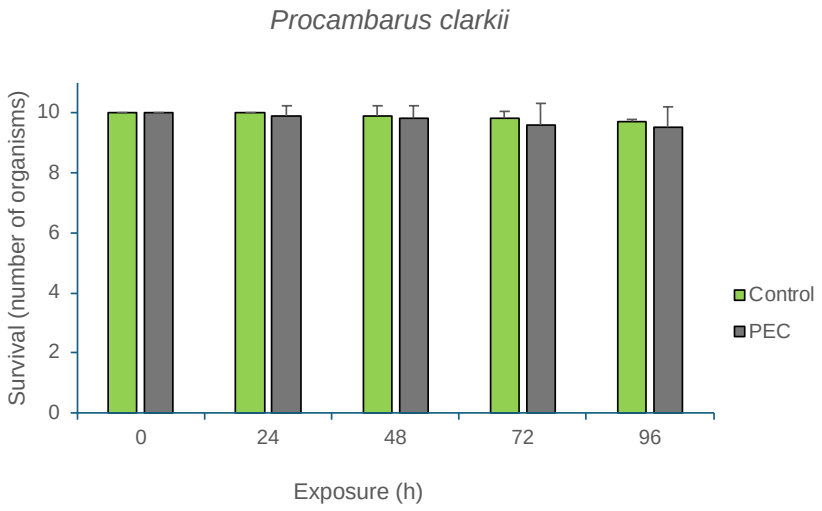


Figure 3.4: Average survival (number of live organisms) ± standard deviation of *P. clarkii* (crayfish) in the control and pesticide mixture (PEC) over 96 hours.

## 3.3 Bioconcentration tests

The primary objective of the bioconcentration tests with pesticide mixtures was to assess the potential for these chemicals to accumulate in aquatic organisms from their surrounding environment. These tests determined the extent to which a pesticide, when present in water, was absorbed and retained by aquatic organisms. The bioconcentration factor (BCF) for each pesticide in the PEC mixture was calculated as the ratio of the pesticide concentration in the organism to its concentration in the test solution (PEC or APW medium). This ratio indicated the chemical's potential to bioaccumulate. Understanding BCF was essential, as pesticides that bioaccumulate could transfer up the food chain, potentially causing toxic effects at higher trophic levels.

The experimental conditions for the bioconcentration tests for each organism were based on the preliminary test results (Section 3.2). The experiment was designed with five replicates per condition (PEC and APW medium: CTR) and was conducted in an incubator chamber under controlled temperature conditions (19±2°C), light intensity (4440-6500 lux), and photoperiod (16L:8D), without food and with an aeration system for oxygenation.

### 3.3.1 Producer, Nasturtium officinale

Five replicates of *N. officinale*, weighing 10-12 g each, were exposed to 500 ml of PEC and APW medium for 96 hours. At the end of the exposure period, yellow leaves were removed, and both the watercress and the medium were collected. The samples were then frozen at -80°C and -20°C, respectively, for subsequent pesticide analysis at WFSR (Wageningen, The Netherlands) and CIEMAT (Madrid, Spain). This experiment was conducted concurrently with the first stage of the two-trophic experiments.

### 3.3.2 Primary Consumer, *Atyaephyra desmaresti*

The primary consumer was exposed to PEC concentrations and a control (APW medium) for 48 hours. Assays were performed in 600 ml of the tested solution, with five replicates for each treatment. Forty *A. desmarestii* individuals were introduced into each vessel. Survival was monitored every 24 hours, and deceased organisms were removed. At the end of the test, surviving *A. desmarestii* and the media were collected from each vessel and frozen for subsequent pesticide analysis.

### 3.3.3 Secondary Consumer, *Procambarus clarkii*

The bioconcentration assessment of *P. clarkii* was conducted after 48 hours of exposure to PEC and APW medium. The assays were carried out in 1000 ml of the tested solution, with five replicates for each treatment. One *P. clarkii* was introduced into each vessel. Mobility and survival were monitored during the exposure period. After 48 hours, at the end of the test, surviving *P. clarkii* and the tested solutions were collected from each vessel and frozen for subsequent pesticide analysis.

**Results**

In these bioconcentration tests with pesticide mixtures, the accumulation potential of the pesticides was confirmed in the three selected aquatic organisms (Figure 3.5). Across both countries studied (Spain and the Czech Republic) and among the three species, there was an increase in the pesticide levels in the organisms when comparing the values from the control and PEC. Watercress, which had the longest exposure time, exhibited the highest potential for bioconcentration. Organisms from the three trophic levels in the Czech Republic showed higher bioconcentration of the tested pesticides.

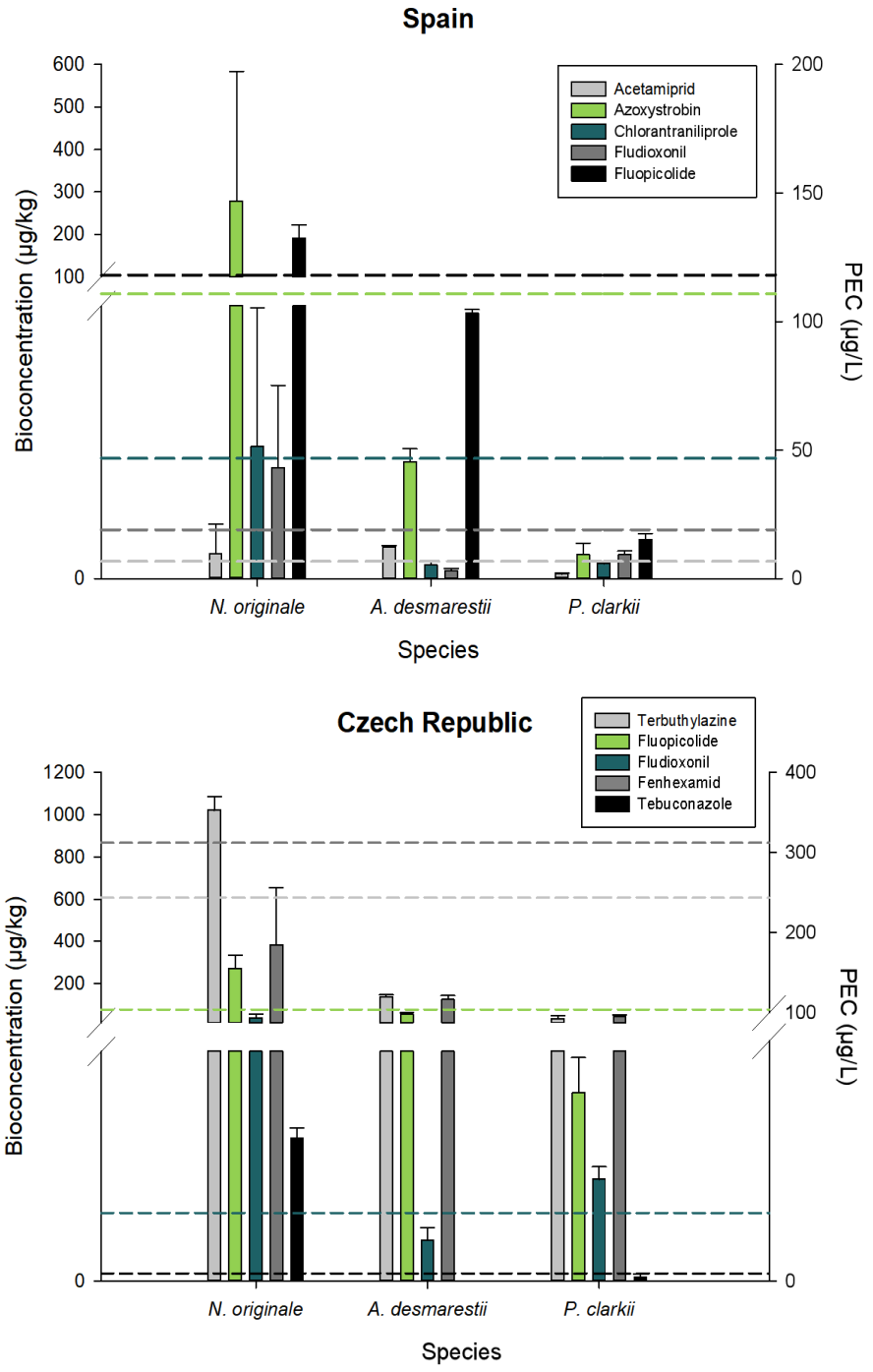


Figure 3.5: Bioconcentration of different pesticides (µg/Kg) in the producer (*N. originale*), primary consumer (*A. desmarestii*) and secondary consumer (*P. clarkii*). Pesticide mixtures from Spain and the Czech Republic were tested.

### Bioconcentration factor

The bioconcentration factor (BCF) is a metric that quantifies the extent to which a substance, such as a pesticide accumulates in an organism´s tissues from the surrounding environment, relative to the concentration in the environment itself. It is calculated as the ratio of the concentration of the substance in the organism (on a mass basis) to its concentration in the surrounding medium (on a volume basis) under steady-state conditions: BCF (L/kg) = Concentration in the organism (μg/kg)/Concentration medium ((μg/L). A BCF greater than one indicates that an organism is accumulating the substance, with a concentration in the organism higher than in the surrounding medium. A very high BCF suggests an increased potential for the substance to accumulate within organisms, which could lead to bioaccumulation and possible biomagnification along the food chain, posing risks to higher trophic levels. Unlike the biomagnification factor, which accounts for trophic transfer, the BCF specifically focuses on direct absorption of the substance from the environment.

The BCF helps quantify the extent to which each pesticide in the PEC mixture from Spain and The Czech Republic is absorbed from the medium into the biomass of the producer *N. officinale*, as well as into the tissues of the primary consumer, *A. desmarestii*, and the secondary consumer, *P. clarkii*.

Table 3.2: Bioconcentration Factor (BCF) (L/kg) for the Producer, *N. officinale* (Watercress), the Primary Consumer, *A. desmarestii* (Shrimp), and the Secondary Consumer, *P. clarkii* (Crayfish). Values in yellow indicate BCF (L/kg) > 1. Grey, blue, and green represent insecticides, fungicides, and herbicides, respectively.

| Country | Pesticides | BCF (L/kg) | | |
|---|---|---|---|---|
| | | *N. officinale* | *A. desmarestii* | *P. clarkii* |
| SPAIN | Acetamiprid | 1.74 | 1.74 | 0.25 |
| | Azoxystrobin | 2.76 | 0.31 | 0.06 |
| | Chlorantraniliprole | 1.15 | 0.11 | 0.12 |
| | Fludioxonil | 1.64 | 0.16 | 0.48 |
| | Fluopicolide | 1.84 | 0.74 | 0.11 |
| The Czech Republic | Terbuthylazine | 4.19 | 0.57 | 0.14 |
| | Fluopicolide | 2.61 | 0.52 | 0.09 |
| | Fludioxonil | 2.73 | 0.15 | 0.37 |
| | Fenhexamid | 1.23 | 0.40 | 0.14 |
| | Tebuconazole | 4.67 | 0.00 | 0.19 |

In the table 3.2 are represented the BCF values for the five pesticides found in each organism after being exposed to the PEC mixtures from Spain and The Czech Republic. Each pesticide's BCF value indicates the extent to which it accumulates in each organism.

In Spain, Acetamiprid, an insecticide, exhibits a relatively high bioconcentration factor (BCF) in both *N. officinale* and *A. desmarestii*, with values reaching 1,74 L/kg, though its concentration is much lower in *P. clarkii*, with a BCF of 0,25 L/kg. However, the BCF decreases significantly in *P. clarkii* (0,25 L/kg) indicating lower accumulation in this higher-level consumer. Azoxystrobin, a fungicide, has the highest BCF observed in *N. officinale*, at 2,76 L/kg among tested pesticides in Spain, but its accumulation decreases sharply in *A. desmarestii* and even further in *P. clarkii*, suggesting a clear trend of reduced bioconcentration at higher trophic levels. Chlorantraniliprole, an insecticide, exhibits BCF higher than 1 in *N. officinale* (1,15 L/kg) but shows very low BCFs in both *A. desmarestii* (0,11 L/kg) and *P.*

*clarkii* (0,12 L/kg. Fludioxonil, a fungicide, shows a unique pattern. While it has a relatively high BCF in *N. officinale* (1,64 L/kg), it drops significantly in *A. desmarestii* (0,16 L/kg) but then increases in *P. clarkii* (0,48 L/kg). This suggests potential biomagnification from *A. desmarestii* to *P. clarkii* for this particular pesticide. As for the fluopicolide, another fungicide, displays a gradual decrease in BCF across trophic levels. It has the second-highest BCF in *N. officinale* (1,84 L/kg), followed by a moderate BCF in *A. desmarestii* (0,74 L/kg), and a low BCF in *P. clarkii* (0,11 L/kg).

As for The Czech Republic, terbuthylazine, a herbicide, shows a high BCF of 4.19 L/kg in *N. officinale*, indicating significant accumulation in this primary producer. However, its BCF decreases substantially in *A. desmarestii* (0,57 L/kg) and further in *P. clarkii* (0,14 L/kg), suggesting reduced bioaccumulation at higher trophic levels. Fluopicolide demonstrates the third-highest BCF (2,61 L/kg) in *N. officinale* among the tested pesticides. Its accumulation decreases in *A. desmarestii* (0,52 L/kg) and drops further in *P. clarkii* (0,09 L/kg), following a similar trend to terbuthylazine. Fludioxonil exhibits a high BCF of 2,73 L/kg in *N. officinale*. Interestingly, it shows a sharp decline in *A. desmarestii* (0,15 L/kg) but then increases in *P. clarkii* (0,37 L/kg), suggesting potential biomagnification from *A. desmarestii* to *P. clarkii* for this particular pesticide. Fenhexamid, also a fungicide, displays moderate accumulation in *N. officinale* (1,23 L/kg) and shows a gradual decrease in BCF across trophic levels, with 0,40 L/kg in *A. desmarestii* and 0,14 L/kg in *P. clarkii*. Tebuconazole, another fungicide, exhibits the highest BCF (4,67 L/kg) in *N. officinale* among all tested pesticides. Surprisingly, it shows no detectable accumulation in *A. desmarestii* (BCF of 0,00 L/kg) but reappears with a low BCF of 0.19 L/kg in *P. clarkii*. This pattern suggests that tebuconazole's bioaccumulation potential decreases at higher trophic levels, possibly due to metabolic processes in consumers. Overall, *N. officinale* consistently shows the highest BCFs for all pesticides, suggesting higher bioconcentration potential in producers. This might indicate that producers may serve as significant reservoirs for these pesticides in the aquatic ecosystem. Generally, BCFs decrease as we move from up the trophic levels (from primary consumer to secondary consumer), with some exceptions (e.g., fludioxonil in *P. clarkii* in Spain, and fludioxonil and

tebuconazole in *P. clarkii* in The Czech Republic), showing a potential limited bio-amplification in consumers. The varying BCFs across species and pesticides highlight the complexity of pesticide dynamics in aquatic ecosystems. The higher accumulation in *N. officinale* suggests that lower trophic level organisms may be at greater risk of pesticide exposure and potential toxicity.

## Conclusions

Bioconcentration tests confirmed significant pesticide accumulation in the three aquatic organisms across Spain and the Czech Republic, with *N. officinale* showing the highest potential due to longer exposure times. Organisms in the Czech Republic exhibited greater pesticide levels overall.

*N. officinale* consistently had the highest bioconcentration factors (BCFs), indicating its role as a reservoir for pesticides. BCFs decreased from *N. officinale* (producer) to *A. desmarestii* (primary consumer) to *P. clarkii* (secondary consumer), suggesting reduced accumulation at higher trophic levels.

In Spain, *acetamiprid* and *azoxystrobin* accumulated notably in *N. officinale*, while *fludioxonil* showed potential biomagnification to *P. clarkii*. In the Czech Republic, *tebuconazole* had a high BCF in *N. officinale* but none in *A. desmarestii*.

These findings indicate that primary producers face higher pesticide toxicity risks, with implications for higher trophic levels. The variability in BCF values highlights the complexity of pesticide dynamics in aquatic ecosystems, underscoring the need for ongoing monitoring and management strategies.

## 3.4 Multispecies microcosm

The objective of this study was to use multispecies microcosms to evaluate the trophic transfer and potential bio-amplification of pesticides within aquatic food webs. By simulating natural ecosystems and incorporating multiple trophic levels, the experiment aims to determine how pesticides move through the food chain, from primary producers, *N. officinale*, to primary (*A. desmarestii*) and to the secondary consumer (*P. clarkii*). This approach provides a realistic assessment of the bioaccumulation potential of contaminants and their amplification at higher trophic levels, offering valuable insights into the ecological risks posed by pesticide exposure in aquatic environments.

The experimental conditions to perform the multispecies microcosm tests, were based on the results from section 3.2 and 3.3. The experiment was conducted in an incubator chamber under controlled temperature conditions (19±2°C), light intensity (4440-6500 lux), with photoperiod ($16^{L}$h:$8^{D}$h) and with an aeration systems oxygen.

### *3.4.1* A two-trophic level aquatic microcosm

The first microcosm setup (Figure 3.7), aimed to investigate the effects of pesticide transfer across three trophic levels in three stages (groups). The experiment was conducted using microcosms arranged in a randomized design, with replicates for each experimental condition. Control microcosms contained only uncontaminated conditions and were not exposed to the pesticide mixture (APW for the stage 1, and APW with uncontaminated organism, producer or primary consumer, for stage 2 and 3, respectively). In contrast, contaminated microcosms were treated with APW and contaminated producer or primary consumer from the previous stage, and PEC with uncontaminated and contaminated organisms from the previous stage.

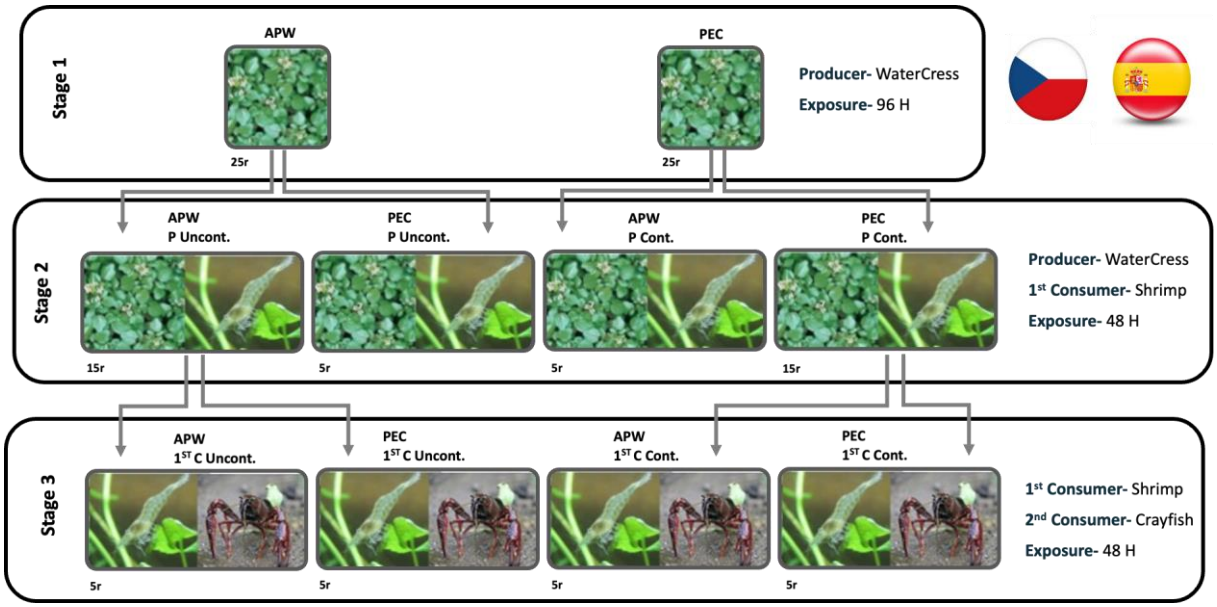


Figure 3.6: Experimental design of the two-trophic level aquatic microcosm. APW (Culture medium)-The CRT condition. PEC (Predicted Environmental Concentration)- The tested condition. Producer (Watercress- *Nasturtium officinale*), 1st consumer (Shrimp- *Atyaephyra desmarestii*), and 2nd consumer (Crayfish- *Procambarus clarkii*). P Uncont. - Producer uncontaminated; P Cont. - Producer contaminated; 1st C Uncont. – Primary consumer uncontaminated; 1st C Cont. – Primary consumer contaminated; 2nd C Uncont. – Secondary consumer uncontaminated; 2nd C Cont. – Secondary consumer contaminated.

**Stage 1- Exposure of primary producer (watercress- *N. officinale*)**

In the first stage of the experiment (Figure 3.6), approximately 10-12 g of watercress (*Nasturtium officinale*) was exposed to 500 ml of control (APW medium) and PEC for 96 hours, with 25 replicates for each condition. After exposure, 5 replicates from each condition (used for the bioconcentration test for *N. officinale*, section 3.3), both uncontaminated and contaminated, were separated and frozen at -80ºC for chemical analysis to assess bioaccumulation of the pesticide mixture at the producer level. The remaining watercress (20 replicates per condition) was transferred to the second stage of the experiment. The APW and PEC from the vessels were also frozen at -20ºC for subsequent pesticide analysis.

### Stage 2- Exposure of primary consumer (shrimp- *A. desmarestii*)

The second stage (Figure 3.6) aimed to evaluate the trophic transfer (bioaccumulation) from the producer (*Nasturtium officinale*) to the primary consumer (shrimp, *Atyaephyra desmarestii*) and the potential for bio-amplification. The assays were conducted in vessels containing 600 ml of either APW medium or PEC, with 40 *A. desmarestii* per vessel, across four experimental conditions (with 5 or 15 replicates each) over a 48-hour exposure period. The experimental setup included: (A) shrimps exposed to APW medium and fed with 5-7 g (sufficient biomass for 48 hours of consumption) of uncontaminated (P Uncont) or contaminated (P Cont.) from the first stage of the experiment, and (B) shrimps exposed to PEC and fed with either uncontaminated or contaminated watercress (PEC + P Uncont. and PEC + P Cont.). This design aimed to assess the transfer of pesticides from the producer to the primary consumer and evaluate the potential transfer to the second trophic level, either through feeding or environmental exposure. Five replicates from each condition (*N. officinale*, *A. desmarestii*, and the mediums APW and PEC) were frozen for pesticide analysis, while an additional 5 replicates (shrimps) from two conditions (APW + P Uncont. [CTR] and PEC + P Cont.) were carried forward to the third stage of the experiment (stage 3, (Figure 3.7).

### Stage 3- Exposure of secondary consumer (crayfish- *P. clarkii*)

The final stage of the experiment (Figure 3.6) focused on assessing trophic transfer (bioaccumulation) and the potential for bio-amplification from the primary consumer (*A. desmarestii* shrimps) to the secondary consumer (crayfish). A single crayfish was exposed for 48 hours to four different conditions: either 1000 ml of a control medium (APW) or a contaminated medium containing a pesticide mixture (PEC) and fed with 20 shrimps (either uncontaminated or contaminated) from the second stage of the experiment. Each of the four treatments was replicated five times, with the following conditions: 1) [CTR] = APW + uncontaminated shrimps + crayfish; 2) PEC + uncontaminated shrimps + crayfish; 3) APW + contaminated

shrimps + crayfish; and 4) PEC + contaminated shrimps + crayfish (Figure 3.6). As in the previous stages, the organisms and media from each condition were frozen for pesticide analysis. In this stage, bioaccumulation from the environment was assessed using the first two conditions, while trophic transfer through feeding from the primary consumer to the secondary consumer was evaluated using the latter two conditions.

**Results**

These experiments were crucial for understanding the transfer of substances to the next trophic level and the conditions under which this occurs. The bioconcentration values obtained from experiments conducted under control conditions (uncontaminated organisms exposed to APW) consistently remained low. The first two graphs display the concentrations of various pesticides in the tissues of producers after 96 hours of exposure (producer exposure), followed by an additional 48 hours representing the exposure of the primary consumer. The bioconcentration values shown for the producers (Figure 3.7) indicate the levels that the primary consumer will ingest.

The next two graphs illustrate the accumulated pesticide levels in the primary consumer, which are then transferred to the secondary consumer, moving up to the next trophic level. The scenario in which the primary consumer ingested a contaminated producer and was exposed to PEC showed the highest bioconcentration levels. This outcome mirrors the situation where the exposure medium was PEC, but the producer was not contaminated. In both cases (Spain and the Czech Republic), the results emphasize the importance of not only contaminated food but also the impact of pesticide mixtures in the environment.

In the primary consumer, the presence of the contaminated organism in APW suggests depuration during the exposure period, with pesticide levels decreasing in the organism. However, this trend is not observed in the secondary consumer. This difference may be linked to the primary consumer's ability to molt during the exposure period, leading to a reduction in pesticide concentration as some may be lost with the exoskeleton.

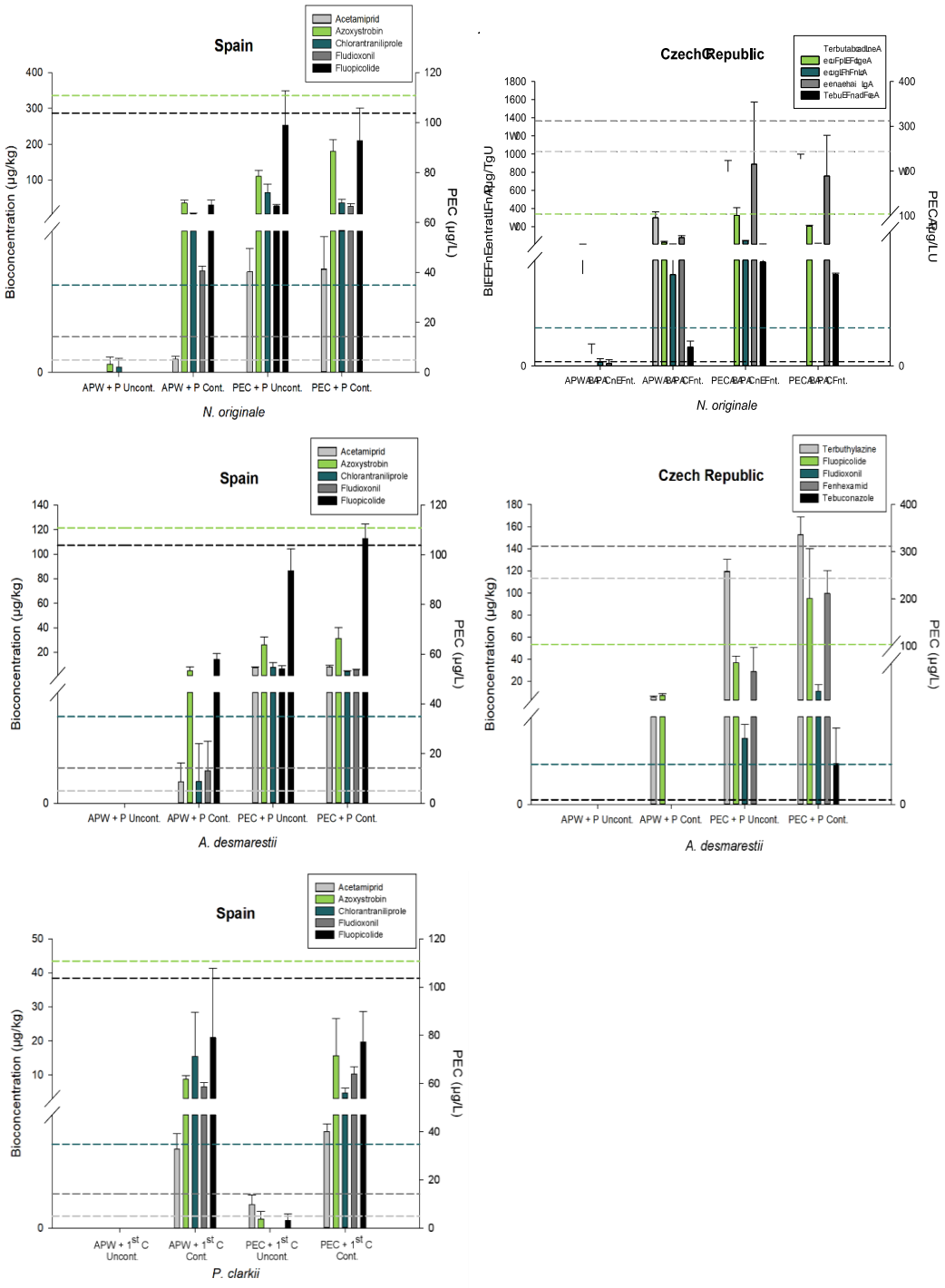


Figure 3.7: Bioconcentration evaluated for the three organisms from different trophic levels that will serve as food at the next trophic level. Different scenarios were tested.

## Bioconcentration factor and Biomagnification factor

Biomagnification refers to the conditions where the substance concentration in an organism exceeds the concentration of its food when the major exposure route occurs from the organism's diet. So, it is used to describe trophic enrichment of substances within food webs and refers to the progressive increase in chemical concentration with increasing organism trophic status. The biomagnification factor (BMF), expressed as a unitless, is calculated as the ratio of the concentration of the substance in the organism (on a mass basis) to the concentration in its diet or prey (on a mass basis) under steady-state conditions: BMF = Concentration in the organism (μg/kg)/Concentration food or prey (μg/kg). A BCF greater than one indicates that biomagnification is occurring, meaning that the substance concentration increases as it moves up the food chain, and a BMF lower than 1 suggest that the substance is not biomagnifying and may even be decreasing as it moves to higher trophic levels.

The two-trophic level microcosm provided insights into distinguishing between trophic transfer occurring via the medium and that occurring through the food route, allowing the calculation of BCFs and BMFs for the various organisms studied.

The results (Table 3.3) from the two-trophic levels microcosm experiment for Stage 2 (*N. officinale* + *A. desmarestii*) provide valuable insights into the bioconcentration and biomagnification patterns of various pesticides in *A. desmarestii* for both Spain and The Czech Republic. In Spain, acetamiprid demonstrates the highest bioconcentration potential with a BCF of 1,49 L/kg, suggesting this pesticide has a significant tendency to accumulate in *A. desmarestii*, via medium. Fluopicolide follows with a BCF lower than 1, 0,83 L/kg, while fludioxonil, azoxystrobin, and chlorantraniliprole show limited bioconcentration with BCFs ranging from 0,23 to 0,44 L/kg. The Czech Republic presents a different profile, with terbuthylazine showing the highest BCF at 0,49 L/kg, though this is still considerably lower than the highest BCF observed in Spain. Fluopicolide has the second-highest BCF at 0,36 L/kg, while fludioxonil and fenhexamid exhibit minimal bioconcentration with very low BCFs of 0,12 and 0,09 L/kg respectively. Notably, tebuconazole shows no detectable bioconcentration in

The Czech Republic. Biomagnification appears to be negligible in both countries. In Spain, BMFs range from 0,01 to 0,08, while in The Czech Republic, they are even lower, ranging from 0,00 to 0,03. The biomagnification factors (BMFs) below 1 indicate that there is no significant trophic transfer of these pesticides from the producer to the primary consumer in the studied food web. This suggests that the accumulation of these pesticides does not increase as they move up the food chain through dietary exposure.

Table 3.3: Bioconcentration factor (BCF (L/kg) and Biomagnification factor (BNF) for the trophic transfer between a producer, *N. officinale* (watercress) and a primary consumer, *A. desmarestii* (shrimp) (Stage 1) and a primary consumer, *A. desmarestii* and the secondary consumer, *P. clarkii* for the two-trophic microcosm. Values in yellow indicates BCF (L/kg) > 1. Grey, blue and green represents the insecticides, fungicides, and herbicides, respectively.

| Two trophic levels microcosm | | Stage 2: (*N. officinale* + *A. desmarestii*) | | Stage 3: (*A. desmarestii*) + *P. clarkii* | |
|---|---|---|---|---|---|
| Country | Pesticide | *A. desmarestii* | | *P. clarkii* | |
| | | BCF (L/kg) | BMF | BCF (L/kg) | BMF |
| SPAIN | Acetamiprid | 1.49 | 0.02 | 0.42 | 0.08 |
| | Azoxystrobin | 0.24 | 0.02 | 0.08 | 0.01 |
| | Chlorantraniliprole | 0.23 | 0.01 | 0.45 | 0.00 |
| | Fludioxonil | 0.44 | 0.01 | 0.45 | 0.00 |
| | Fluopicolide | 0.83 | 0.08 | 0.09 | 0.00 |
| The Czech Republic | Terbuthylazine | 0.49 | 0.01 | 0.17 | 0.00 |
| | Fluopicolide | 0.36 | 0.03 | 0.33 | 0.00 |
| | Fludioxonil | 0.12 | 0.00 | 0.32 | 0.00 |
| | Fenhexamid | 0.09 | 0.00 | 0.13 | 0.13 |
| | Tebuconazole | 0.00 | 0.00 | 0.14 | 0.00 |

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The data presented in Table 3.3, for the two-trophic levels microcosm experiment for Stage 3 (*A. desmarestii* + *P. clarkii*) provide valuable insights into the bioconcentration and biomagnification patterns of various pesticides in the aquatic ecosystem, specifically focusing on trophic transfer between a primary consumer and a secondary consumer, *P. clarkii* in Spain and The Czech Republic. The BCFs and BMF offer a quantitative assessment of how these pesticides accumulate in *P. clarkii* tissues and transfer through the medium and food. The BCF values, ranging from 0,08 to 0,45 L/kg, indicate a relatively low tendency for these pesticides to concentrate in *P. clarkii* tissues compared to their levels in the surrounding water. This suggests that *P. clarkii* may have efficient mechanisms for metabolizing or excreting these compounds, or that the pesticides themselves have properties that limit their accumulation in biological tissues. The BMF values, which are consistently very low (0,00 to 0,13) across both countries, reveal that these pesticides are not biomagnifying as they move up the food chain from *A. desmarestii* to *P. clarkii* through food route. This is a positive finding from an ecological perspective, as it indicates that these contaminants are not becoming more concentrated at higher trophic levels, which could potentially lead to more severe ecological impacts. The variation in BCF and BMF values between different pesticides and between the two countries highlights the complexity of pesticide behavior in aquatic ecosystems. Factors such as the chemical properties of the pesticides, environmental conditions, and species-specific physiological differences may all contribute to these variations. Overall, these results suggest that the studied pesticides pose a relatively low risk of bioaccumulation and biomagnification in this particular food chain. However, it's important to note that this doesn't preclude other potential ecological effects of these pesticides, and continued monitoring and research would be beneficial for a comprehensive understanding of their environmental impact.

**Conclusions:**

The experiments provided valuable insights into the transfer of pesticides across trophic levels in aquatic ecosystems. Results from the first stage indicated that pesticide concentrations in producer tissues directly influence the levels ingested by the primary consumer. The second stage revealed that the highest bioconcentration occurred when the primary consumer ingested a contaminated producer while exposed to the predicted environmental concentration (PEC), highlighting the importance of both contaminated food sources and pesticide mixtures in the environment.

Bioconcentration factors (BCFs) showed varying potential for pesticide accumulation in the primary consumer (*A. desmarestii*), with acetamiprid exhibiting the highest BCF in Spain. In contrast, the Czech Republic's highest BCF was for terbuthylazine, but overall BCFs were lower than those observed in Spain. Biomagnification factors (BMFs) were consistently low across both countries, indicating no significant trophic transfer of pesticides from the primary to the secondary consumer (Procambarus clarkii).

The findings suggest that, despite the presence of these pesticides, their risk of bioaccumulation and biomagnification within this specific food chain is low. This is a positive ecological outcome, as it implies minimal risk of increased concentrations at higher trophic levels. However, the variability in pesticide behavior emphasizes the need for further research to fully understand their environmental impact, as other ecological effects may still occur.

### 3.4.2 A three-trophic level aquatic microcosm

A second experiment was carried out to gain a deeper understanding of the effects of pesticides, not just on individual species but using a multispecies approach that examines the impact of pesticides on multiple species simultaneously. These multispecies assays hold greater ecological relevance as they more closely mimic natural environments compared to single-species tests, providing valuable insights into how stressors influence entire ecosystems.

In this set of microcosms (Figure 3.8), *N. officinale*, *A. desmarestii*, and *P. clarkii* from three different trophic levels were placed in an aquarium for 72 hours in specific proportions: 5-7 grams of *N. officinale*, 40 *A. desmarestii*, and one *P. clarkii*. Two types of mediums were used: a 1000 ml contaminated medium containing a pesticide mixture (PEC) and a non-contaminated medium (APW medium). After the experiment concluded, both the organisms and the mediums were frozen for subsequent pesticide analysis. This setup mimics natural conditions and, when compared to previous experimental results, provides insights into how pesticides are transferred through trophic levels.

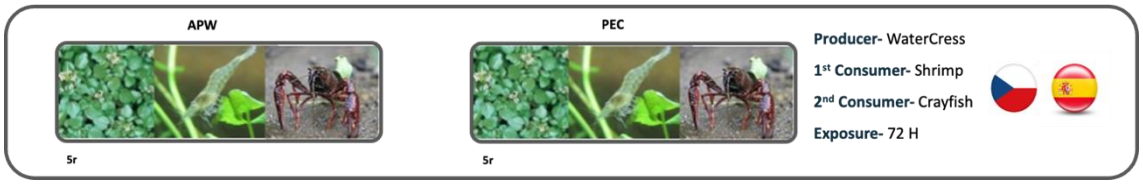


Figure 3.8: Experimental design of the three-trophic level aquatic microcosm. APW (Culture medium)-The CRT condition. PEC (Predicted Environmental Concentration)- The tested condition. Producer (Watercress- *Nasturtium officinale*), primary consumer (Shrimp- *Atyaephyra desmarestii*), and secondary consumer (Crayfish- *Procambarus clarkii*).

## RESULTS

This multispecies approach assessed the impact of pesticides on all three species simultaneously, showing a trend similar to that observed in the bioconcentration test (Figure 3.9). However, for both the Spanish and The Czech pesticide mixtures, the bioconcentration test yielded higher values. This outcome was anticipated, as the bioconcentration test involves a greater number of stress factors.

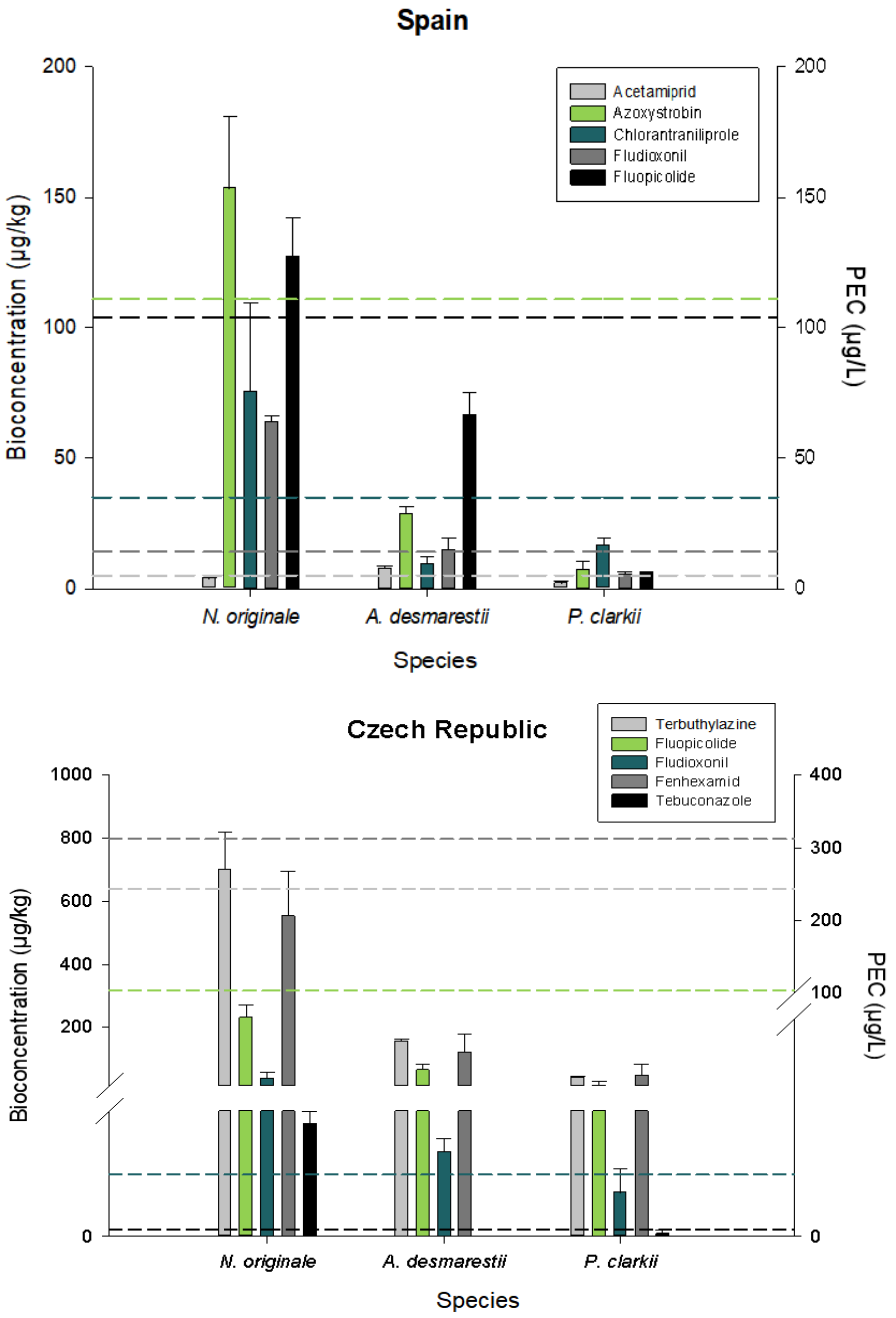


Figure 3.9: Three trophic level aquatic microcosms for Spain and The Czech Republic. Bioconcentration (µg/Kg) for the producer (watercress), primary consumer (shrimp) and secondary consumer (crayfish) placed together in an aquarium for 72 hours.

## Bioconcentration factor

The results from the three-trophic level microcosm experiment provide insight into the bioconcentration patterns of each organism in a coexisting community, closely mimicking the dynamics observed in natural aquatic ecosystems. This setup allows for a more accurate representation of pesticide accumulation across different trophic levels under realistic environmental conditions.

The bioconcentration factors (BCFs) for the three-level microcosm for Spain and The Czech Republic are presented in Table 3.4.

Table 3.4: Bioconcentration factor (BCF (L/kg) for the producer, *N. officinale* (watercress), primary consumer, *A. desmarestii* (shrimp) and secondary consumer, *P. clarkii* (crayfish) for the three-trophic microcosm. Values in yellow indicates BCF (L/kg) > 1. Grey, blue and green represents the insecticides, fungicides, and herbicides, respectively.

| Country | Pesticide | BCF (L/kg) | | |
|---|---|---|---|---|
| | | *N. officinale* | *A. desmarestii* | *P. clarkii* |
| SPAIN | Acetamiprid | 0.76 | 1.57 | 0.40 |
| | Azoxystrobin | 1.38 | 0.26 | 0.07 |
| | Chlorantraniliprole | 2.16 | 0.27 | 0.48 |
| | Fludioxonil | 4.40 | 1.04 | 0.49 |
| | Fluopicolide | 1.22 | 0.64 | 0.06 |
| The Czech Republic | Terbuthylazine | 2.88 | 0.65 | 0.16 |
| | Fluopicolide | 2.22 | 0.63 | 0.16 |
| | Fludioxonil | 2.82 | 0.38 | 0.20 |
| | Fenhexamid | 1.77 | 0.39 | 0.16 |
| | Tebuconazole | 4.49 | 0.00 | 0.13 |

In Spain, the producer, *N. officinale* generally shows the highest BCFs for most pesticides, ranging from 0,76 to 4,40 L/kg. This suggests that *N. officinale* may serve as a significant reservoir for these pesticides in the aquatic ecosystem. *A. desmarestii* shows variable BCFs, ranging from 0,26 to 1,57 L/kg. Interestingly, it has the highest BCF for acetamiprid among all species. *P. clarkii* generally shows the lowest BCFs, ranging from 0,06 to 0,49 L/kg, indicating less bioconcentration at this trophic level. Fludioxonil shows the highest overall BCF (4,40 L/kg) in *N. officinale*, suggesting it has the greatest potential for bioconcentration among the tested pesticides. Acetamiprid exhibits an unusual pattern, with the highest BCF in *A. desmarestii* (1,57 L/kg) rather than *N. officinale*, which is atypical compared to the other pesticides. Fluopicolide and azoxystrobin show relatively low BCFs across all species, suggesting lower bioconcentration potential. Generally, BCFs decrease as we move up the trophic levels (from *N. officinale* to *P. clarkii*), with some exceptions (e.g., acetamiprid). This trend suggests that biomagnification is not a common occurrence for these pesticides in this food web. The decrease in BCFs at higher trophic levels could be due to various factors, including metabolism, excretion, or growth dilution in the higher-level consumers.

The BCFs for the pesticides mixture from The Czech Republic reveal interesting patterns of bioconcentration across trophic levels. The data shows a clear trend of higher bioconcentration in *N. officinale* compared to *A. desmarestii* and *P. clarkii* for all pesticides examined. *A. desmarestii* and *P. clarkii* exhibited similar, lower BCFs for most pesticides, with values generally below 1 L/kg. This indicates a lower tendency for these species to accumulate the studied pesticides, possibly due to differences in habitat, feeding behavior, or metabolic processes. This suggests that *N. officinale*, likely occupying a lower trophic level, has a greater capacity to accumulate these pesticides from the environment. As for the pesticides-specific patterns, tebuconazole exhibited the highest BCF in *N. officinale* at 4,49 L/kg, indicating it has the greatest potential for bioaccumulation among the pesticides studied. Interestingly, tebuconazole was not detected in *A. desmarestii* (BCF of 0,00 L/kg), suggesting possible metabolic differences between species. Terbuthylazine, fluopicolide, and fludioxonil showed BCFs higher than 1, in *N. officinale*, ranging from 2,22 to 2,88 L/kg. These pesticides demonstrated lower

but consistent BCFs in *A. desmarestii* and *P. clarkii*. Fenhexamid had the lowest BCF in *N. officinale* (1,77 L/kg) among the pesticides studied, but still showed higher accumulation compared to the other two species.

## Conclusions

In both countries, the primary producer, *N. officinale*, consistently demonstrated the highest BCFs for most pesticides. The BCFs generally decreased moving up the food chain to *A. desmarestii* and *P. clarkii*, indicating that biomagnification is not a common occurrence for these pesticides in these food webs.

Fludioxonil and tebuconazole exhibited the highest BCFs in *N. officinale* for Spain and The Czech Republic, respectively, suggesting a high potential for bioaccumulation. As for acetamiprid in Spain, showed an atypical pattern with the highest BCF in *A. desmarestii*, contrary to the general trend. Fluopicolide and azoxystrobin demonstrated relatively low BCFs across all species in Spain, indicating lower bioconcentration potential.

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The variability in BCFs among species and pesticides underscores the complexity of pesticide dynamics in aquatic ecosystems. These findings emphasize the need for ongoing research to further understand the ecological implications of pesticide accumulation and its potential effects on aquatic communities.

# 4 General conclusions

The terrestrial microcosm experiment revealed clear trends of the tested pesticides on the bioaccumulation. In earthworms, boscalid and dimoxystrobin showed a potential for bioaccumulation, with BAF values above 1 from the MEC exposure. Earthworms are an important part of the terrestrial food web, providing food for several organisms, including mammals and birds. Therefore, biomagnification in predators of the tested pesticides could be possible. Boscalid and dimoxystrobin, for example, have high toxicity to mammals in short-term dietary tests, with NOEL of 5 mg/kg, according to the PPDB database.

Lettuce roots showed a similar pattern to that of earthworms. Boscalid and dimoxystrobin had the highest bioconcentration factors (BCF), however, azoxystrobin also showed BCF above 1 in roots. Lettuce leaves, on the other hand, showed much lower bioconcentration potential, indicating a limited translocation from roots to leaf. The concentrations detected in the leaves were all below MRL after 56 days of exposure. Based on these results, it can be concluded that the pesticide mixture tested is not likely to pose any risk to consumers. Overall, the results from the terrestrial microcosm experiment indicated a greater concern for environmental exposure, with the potential bioaccumulation in earthworms, and a lesser concern for human exposure, with low bioconcentration in lettuce leaves.

In the aquatic microcosm experiment, bioconcentration tests indicated significant pesticide accumulation in three aquatic organisms across Spain and the Czech Republic, with *N. officinale* exhibiting the highest potential due to longer exposure times. Organisms in the Czech Republic displayed higher overall pesticide levels. BCFs were highest in *N. officinale*, decreasing in *A. desmarestii* and *P. clarkii*, suggesting reduced accumulation at higher trophic levels. In Spain, acetamiprid and azoxystrobin notably accumulated in *N. officinale*, while fludioxonil showed potential biomagnification to *P. clarkii*. In the Czech Republic, tebuconazole had a high BCF in *N. officinale* but none in *A. desmarestii*. BMFs were consistently low across both countries, indicating minimal trophic transfer of pesticides from

primary to secondary consumers. These findings suggest that while primary producers are at greater risk for pesticide toxicity, the overall risk of bioaccumulation and biomagnification within this food chain is low.

Further research is essential to fully understand the ecological implications of pesticide accumulation, as well as to evaluate the broader impacts on biodiversity and ecosystem health in both terrestrial and aquatic systems.

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