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Effects of realistic pesticide mixtures on wheat (*Triticum aestivum*) and lettuce (*Lactuca sativa*)

Paula S. Tourinho[1,*], Vera Silva[2], Paula Harkes[2], Trine Nørgaard[3], Virginia Aparicio[4], and Jakub Hofman[1]

[1]RECETOX, Faculty of Science, Masaryk University, Brno, Czech Republic
[2]Soil Physics and Land Management Group, Wageningen University, Wageningen, the Netherlands
[3]Department of Agroecology, Aarhus University, Tjele, Denmark
[4]Instituto Nacional de Tecnología Agropecuaria, Balcarce, Argentina
*Corresponding author: Paula S. Tourinho, Email: paula.tourinho@recetox.muni.cz

Abstract

The application of pesticides often results in exposure and risks to nontarget organisms. Among these organisms are nontarget terrestrial plants, which are essential for maintaining healthy ecosystems. Despite the rigorous risk assessment process for approving pesticides used in Europe and many developed countries, the impacts of environmentally realistic mixtures remain insufficiently understood. This study evaluated the effects of 11 realistic mixtures on two plant species. Each mixture comprised five pesticides selected by their estimated risk to terrestrial plants (from regulatory reports) and their frequency of detection in agricultural soils across 10 European countries and Argentina. Two standardized assays were conducted with wheat (*Triticum aestivum*) and lettuce (*Lactuca sativa*): A root growth inhibition test (International Organization for Standardization 11269-1) and a seedling emergence and growth test (Organisation for Economic Co-operation and Development Test Guideline 208). Three concentrations were used: Median measured environmental concentration, predicted environmental concentration, and 5-times the predicted environmental concentration. Significant effects were observed for 10 of the 11 mixtures, and these were species and endpoint specific, likely reflecting variation in mixture composition and herbicide modes of action. Root growth was more sensitive than seedling emergence and shoot biomass. Considering the median measured and predicted environmental concentration treatments, root growth was affected in nine mixtures for both species, while emergence and biomass were affected in five and seven mixtures, respectively. These results indicate that realistic combinations of pesticide residues can cause deleterious effects on terrestrial plants even at concentrations below predicted environmental thresholds, where no adverse impact would ordinarily be expected. Given that current European risk assessments emphasize spray drift and single-compound exposures, consideration of mixture effects is crucial for adequately evaluating risks to nontarget terrestrial plants.

Keywords nontarget terrestrial plants, plant protection products, soil ecotoxicology, predicted environmental concentration

Introduction

The extensive use of pesticides, particularly herbicides, can have detrimental effects on nontarget plant species (NTTPs). The European Food Safety Authority (EFSA) Panel on Plant Protection Products and Their Residues (EFSA PPR Panel, 2014) defines NTTPs broadly as “all plants growing outside fields, and those growing within fields that are not the intended pesticide target,” encompassing wild plants in surrounding habitats and noncrop plants within agricultural fields. Since NTTPs are a major part of natural and seminatural vegetation, their harm directly contributes to wider plant biodiversity loss. Research consistently links pesticide applications to decline in wild plant diversity (Geiger et al., 2010; Schmitz et al., 2014), highlighting the importance of assessing pesticide impacts on vegetation. As primary producers, terrestrial plants form the foundation of terrestrial food webs, contribute to nutrient cycling through stimulation of microbial activity (Hamilton & Frank, 2001), and improve key soil characteristics (Massaccesi et al., 2015). Understanding how pesticides affect NTTPs is therefore essential for protecting ecosystems’ functioning and soil health.

Several standard protocols are available for testing the effects of chemicals on NTTPs, including measurements of seed germination, root and seedling growth in biomass or length, visual phytotoxicity, and reproductive parameters (Mendes da Silva & Andrade-Vieira, 2025). The approval process for active substances in plant protection products at the European level, along with the national authorization of plant protection products

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within European Union member states, requires ecotoxicological testing on terrestrial plants, conducted using the Organisation for Economic Co-operation and Development (OECD) Test Guideline 208 ("Seedling Emergence and Growth Test"; European Commission, 2002). These tests are typically conducted with 6 to 10 plant species representing different taxonomic groups. Effects on terrestrial plants ≥50% for at least one species at the maximum application rate are considered unacceptable (European Commission, 2002).

In environmental risk assessment, exposure methods aim to replicate realistic conditions, with foliar application being the most common approach. The reason is that pesticide drift from nearby application areas is expected to represent the main pathway through which NTTPs are unintentionally exposed (European Commission, 2002). However, pesticide residues in soil may also represent an important and often overlooked source of exposure. Several studies have reported the widespread presence of multiple pesticide residues in agricultural soils, originating from previous applications or environmental contamination (Froger et al., 2023; Geissen et al., 2021; Hvězdová et al., 2018; Knuth et al., 2024). Nontarget plant species growing in or near agricultural areas may be continuously exposed to these residues through root uptake. This route of exposure is not adequately addressed in current regulatory testing frameworks.

A further limitation of current environmental risk assessment approaches is the limited consideration of pesticide mixtures (Gruss et al., 2025). Environmental risk assessment focuses on individual substances, and to our knowledge, only a limited number of studies have tested the toxicity of pesticide mixtures on terrestrial plants (e.g., Cedergreen et al., 2007; Gruss et al., 2025; Pitombeira de Figueirêdo et al., 2020; Santos et al., 2011; Zhang et al., 1995). Moreover, these studies addressed mostly binary mixtures, which are far from the reality of multiresidue occurrence in agroecosystems (Knuth et al., 2024; Tourinho, Hochmanová, Kukučka, Hronová, et al., 2025). From that point, the current risk assessment approaches may underestimate real-world exposure scenarios.

To address these regulatory gaps, we investigated the effects of multiresidue pesticide mixtures commonly detected in agricultural soils from 10 European countries and Argentina (AR) on two NTTPs: One monocotyledon and one dicotyledon. A total of 11 mixtures were tested, each composed of five pesticides. The mixtures were selected by pesticide residues commonly found in agricultural soils, considering their frequency in soil samples (Knuth et al., 2024) and their phytotoxicity. Root growth test and seedling emergence and growth test were conducted using wheat (*Triticum aestivum*) and lettuce (*Lactuca sativa*) as test species. Although based solely on nominal concentrations, our results are expected to be especially relevant in applied ecotoxicology, particularly for risk assessors, but they also intend to create a foundation for larger, mechanistic studies.

Material and methods

Selection of pesticide mixtures

This study is embedded in the SPRINT project, where a sampling campaign was conducted in case study sites from 11 countries. The pesticide mixtures to test, one per case study site/country, were selected using a prioritization approach described by Jegede et al. (2025), in which pesticides found in agricultural soils were scored according to their frequency of detection in soils and their estimated risk to terrestrial plants.

The dataset underpinning this prioritization originates from Knuth et al. (2024), who carried out a comprehensive analysis of pesticide residues in agricultural soils. In that study, 201 soils were sampled from 10 European countries, covering conventional and organic farms and a range of cropping systems. In our study, soil samples from AR were also included; therefore, a total of 215 soil samples were considered. Each soil sample was analyzed for 192 pesticides: 50 herbicides, 57 fungicides, 45 insecticides, 39 metabolites, and 1 synergist. A total of 100 distinct pesticide residues were detected above limits of quantification in soil samples. For the mixture selection, pesticides banned before the soil sampling season were excluded from prioritization, to focus only on pesticides currently in use.

The risk to terrestrial plants was evaluated via risk quotients, a simple ratio between exposure and toxicity data, retrieved or derived from EFSA reports (see online supplementary material Table S1). Data for active substances were used whenever available; when absent, data from the corresponding formulations were applied. For metabolites lacking exposure data, exposure was estimated by multiplying the predicted environmental rate in edge of field (PERoff-field) of the parent compound by the percentage of the metabolite formation.

The next step in the scoring process, repeated for each country, involved multiplying the frequency of detection of pesticides by their risk quotient. These values were normalized for equal weights in the scoring. The frequency of detection was transformed to the percentage of occurrence in soil samples (0 to 1). The risk quotient was normalized by dividing all values by the maximum value obtained in the country so that the highest risk quotient value is 1. The five pesticides with the highest score (frequency of detection × risk quotient) were selected for the mixture in each country. The number 5 was chosen as a representative of the most common number of pesticide residues found in agricultural soils (Geissen et al., 2021; Hvězdová et al., 2018; Knuth et al., 2024; Kosubová et al., 2020; Pelosi et al., 2021; Silva et al., 2019). In total, 15 herbicides, 11 fungicides, 3 insecticides, and 3 metabolites were selected (Table 1). For the calculation of risk quotient and scoring system, see online supplementary material SM2.

Soil spiking procedure

The same type of soil was used in all assays, an agricultural soil collected from Unifarm (Wageningen University, the Netherlands [NL]). The soil characteristics were as follows: 6.2 ± 0.1 pH_{CaCl2}, 0.6% soil total organic carbon, 1% clay, 8% silt, and 89% sand. The soil was air-dried, sieved at 2 mm, and shipped to Brno (Czech Republic [CZ]), where it was kept at room temperature. This soil was selected because the low organic matter content typical of agricultural soils can lead to increased pesticide bioavailability (van Gestel, 2012).

The soil was spiked at three concentrations (Table 1). The first concentration was the median measured environmental concentration (MEC) of each pesticide in the country

Table 1 Pesticide mixture composition as prioritized for 11 countries surveyed in the SPRINT project and their concentrations tested in this study.

Country: Pesticide	Class	MEC	PEC	5PEC
Argentina				
Glyphosate	Herbicide	0.54	5.76	28.8
AMPA	Metabolite	1.33	2.04	10.2
Diflufenican	Herbicide	0.01	0.25	1.25
2,4-D	Herbicide	0.02	0.75	3.75
Lambda-cyhalothrin	Insecticide	0.01	0.03	0.16
Switzerland				
AMPA	Metabolite	0.34	2.04	10.2
Difenoconazole	Fungicide	0.01	0.14	0.68
Myclobutanil	Fungicide	0.01	0.67	3.36
Emamectin	Insecticide	0.01	0.33	1.65
Tebuconazole	Fungicide	0.01	0.19	0.93
Czech Republic				
AMPA	Metabolite	0.14	2.04	10.2
Diflufenican	Herbicide	0.01	0.25	1.25
Mecoprop P	Herbicide	0.01	1.60	8.00
Dimethenamid (P)	Herbicide	0.02	1.15	5.76
Deltamethrin	Insecticide	0.03	0.02	0.11
Denmark				
AMPA	Metabolite	0.12	2.04	10.2
Diflufenican	Herbicide	0.02	0.25	1.25
Fluopyram	Herbicide	0.01	0.26	1.30
Pendimethalin	Herbicide	0.05	2.13	10.6
Boscalid	Fungicide	0.01	0.40	1.98
Spain				
AMPA	Metabolite	0.11	2.04	10.2
Glyphosate	Herbicide	0.08	5.76	28.8
Metazachlor	Herbicide	0.14	1.33	6.67
Propyzamide	Herbicide	0.02	2.00	10.0
Difenoconazole	Fungicide	0.02	0.14	0.68
France				
AMPA	Metabolite	0.87	2.04	10.2
Glyphosate	Herbicide	0.26	5.76	28.8
Fenbuconazole	Fungicide	0.01	0.47	2.34
Metrafenone	Fungicide	0.04	0.39	1.97
Difenoconazole	Fungicide	0.01	0.14	0.68
Croatia				
Glyphosate	Herbicide	0.17	5.76	28.8
AMPA	Metabolite	0.52	2.04	10.2
Deltamethrin	Insecticide	0.04	0.02	0.11
Trifloxystrobin	Fungicide	0.01	0.50	2.50
Tebuconazole	Fungicide	0.07	0.19	0.93
Italy				
Propyzamide	Herbicide	0.01	2.00	10.0
Pendimethalin	Herbicide	0.04	2.13	10.6
Lambda-cyhalothrin	Insecticide	0.01	0.03	0.16
Oxyfluorfen	Herbicide	0.05	1.92	9.60
Difenoconazole	Fungicide	0.01	0.14	0.68
The Netherlands				
Metribuzin	Herbicide	0.04	1.40	7.00
Metobromuron	Herbicide	0.18	2.67	13.3
AMPA	Metabolite	0.09	2.04	10.2
Diflufenican	Herbicide	0.01	0.25	1.25
Prosulfocarb	Herbicide	0.06	5.33	26.6

(continued)

Table 1 (continued)

Country: Pesticide	Class	MEC	PEC	5PEC
Portugal				
Glyphosate	Herbicide	0.79	5.76	28.8
AMPA	Metabolite	1.91	2.04	10.2
Iprovalicarb	Fungicide	0.17	0.40	1.99
Penconazole	Fungicide	0.02	0.14	0.71
Metolachlor oxanilic acid	Metabolite	0.04	0.40	2.01
Slovenia				
Metolachlor (S)	Herbicide	0.04	1.92	9.60
Terbuthylazine	Herbicide	0.01	1.13	5.63
Thiencarbazone-methyl	Herbicide	0.04	0.06	0.30
AMPA	Metabolite	0.07	2.04	10.2
Terbuthylazine desethyl	Metabolite	0.01	0.33	1.63

Note. The MEC, PEC, and 5PEC are given in mg kg^{-1} dry soil. MEC = median measured environmental concentration; PEC = predicted environmental concentration; 5PEC = 5-times PEC; AMPA = aminomethylphosphonic acid; 2,4-D = 2,4-dichlorophenoxyacetic acid.

(Knuth et al., 2024). The second concentration was the highest predicted environmental concentration (PEC) retrieved from EFSA reports, and the third concentration was set as 5 times the PEC values (5PEC). The later concentration was used as the worst-case scenario. For every country, a single batch of spiked soil was prepared, and both plant species were tested simultaneously. Negative and solvent controls were also conducted by adding water and a water/acetone mixture, respectively. Some countries were tested in pairs and therefore sharing the controls, while others were tested individually.

Stock solutions of the pesticides were prepared in acetone, except for glyphosate, aminomethylphosphonic acid (AMPA), and metolachlor oxanilic acid, which were prepared in distilled water. Prior to addition, the soil was premoistened to 40% of its maximum water holding capacity (see online supplementary material Figure S1). The volume of stock solutions necessary to achieve the final soil concentrations was pipetted into a beaker, and pure acetone was added to reach a total volume of 80 ml of solution (to add the same amount of acetone in all treatments). This volume was selected to ensure a uniform distribution of added substances throughout the soil. The acetone solutions (80 ml) were mixed into 10% portions of soil (i.e., 700 g) from each treatment and left to evaporate in the fume hood for 24 hr. Pure acetone (80 ml) was mixed in a 10% portion of the solvent control soil. A 10% portion of negative control soil was also placed in the fume hood and used to determine the water loss during the solvent evaporation. After replenishing the water loss, the 10% portions of soil were mixed with the 90% remaining soil and moistened to 60% water holding capacity. For the mixtures containing water-soluble pesticides (glyphosate, AMPA, or metolachlor oxanilic acid), the pesticides were dissolved in water and added as necessary to adjust to 60% water holding capacity.

Ecotoxicology tests

One monocotyledonous species (*T. aestivum*) and one dicotyledonous species (*L. sativa*) were selected for the phytotoxicity tests. The root growth inhibition test and seedling emergence and growth test were conducted following guidelines from the International Organization for Standardization (2012; ISO 11269-1) and OECD (2006; Test No. 208), respectively.

For the root growth inhibition test (International Organization for Standardization, 2012), seeds were pregerminated in Petri dishes containing filter paper soaked in distilled water at 23 °C in the dark for 24 hr. Fifteen germinated seeds were sown into plastic boxes (15 × 25 cm) containing approximately 200 g of freshly spiked soil. Five boxes were prepared per treatment, with each box considered an individual replicate. Plants were grown in a greenhouse at a temperature of 22 ± 10 °C and a 16:8-hr light: dark photoperiod. After 5 days, plants were removed from the boxes, gently washed, and patted dry. The root size was measured with a ruler.

For the seedling emergence and growth test (OECD, 2006), 10 seeds were planted in glass pots containing approximately 500 g of freshly spiked soil. Five pots were prepared per treatment, with each pot considered an individual replicate. Pots were placed in a greenhouse at a temperature of 22 ± 10 °C and a 16:8-hr light: dark photoperiod. The soil moisture content was replenished regularly (three times a week). Pots were checked daily for plant emergence and the test run for 14 to 21 days after at least 50% of the plants had emerged in the controls. The number of emerged seeds was recorded, and shoots were harvested at the surface of the soil and washed. The shoots were dried at 60 °C for 24 hr and weighed, and the dry biomass was recorded.

Data analysis

Root length of the 15 plants was averaged within each replicate, and the mean of the five replicate values was calculated for each treatment. Seedling emergence and shoot biomass of the 10 plants were recorded as the total number of emerged plants and total weight of surviving plants per replicate, and the mean of the five replicate values was calculated for each treatment. Coefficients of variation were calculated for control treatments.

Statistical analyses were performed separately for each plant species and endpoint (root length, seedling emergence, and shoot biomass). All statistical analyses were conducted on untransformed raw data. A Kruskal–Wallis test, followed by

Tukey-type nonparametric multiple comparisons, was used to compare treatment effects (control, solvent control, MEC, PEC, and 5PEC), defined by nominal concentrations within each mixture. The nonparametric tests were used because the data failed to meet normality assumptions (Shapiro–Wilk test), even after transformation. The differences among the mixtures (countries) were not statistically tested, since mixtures differed in chemical composition and were therefore not directly comparable. Statistical significance was defined as $p < 0.05$. For visualization, results were expressed as a percentage of the solvent control and standard deviation of replicates (mean ± *SD*).

A principal component analysis (PCA) was conducted to reveal the relationships among mixtures, tested parameters, and concentrations. Prior to analysis, numeric variables were centered and standardized to *z* scores. The suitability of the dataset for PCA was assessed by the Kaiser–Meyer–Olkin measure and Bartlett's test of sphericity. For PCA visualization, replicate values ($n = 5$) were averaged to obtain a single centroid per mixture and treatment level, ensuring equal weighting of all treatments in the analysis.

All analyses were conducted in R. Tukey-type multiple comparisons were performed by the nparcomp package, and PCA was carried out per the pcaMethods package.

Results

Root growth inhibition test

In the root growth test, germination rates in controls exceeded 90%, and the coefficient of variation in control groups remained <20% for both species.

The effects on root growth inhibition are shown in Figure 1 (for heat map, see online supplementary material Figure S2). In wheat, significant root length reductions occurred in 9 of the 11 mixtures as compared with the solvent control. Inhibitions at environmentally relevant concentrations (MEC) were detected for 3 mixtures: Switzerland (CH; 63.5% of solvent control), Spain (ES; 57.6%), and Slovenia (SI; 68.7%). Eight mixtures showed strong inhibitions at PEC (9.1%–69.7%) and 5PEC (4.6–71.7%), while the Portugal (PT) mixture showed a no dose–related effect, with significant effects only at PEC (83.6%).

In lettuce, root length decreased significantly in seven mixtures, with effects at MEC in four mixtures: AR (54.5%), Denmark (DK; 84.1%), ES (60.5%), and SI (41.9%). In addition to these four mixtures, three others (CZ, NL, and PT) showed effects at PEC (8.6%–76.4%) and 5PEC (0%–77.9%). An unexpected pattern occurred in the mixture from Croatia (HR), where root length increased at MEC and PEC in both species.

Seedling emergence and growth

The seedling emergence and growth test met the validity criteria for control treatments according to Test No. 208 guidelines (OECD, 2006): ≥70% seedling emergence in controls and ≥90% survival of emerged seedling, no visual phytotoxicity, and normal growth and morphology variation among plants. The coefficient of variation in controls was always <20% for wheat, although it ranged from 21% to 26% in three controls for lettuce.

Effects on seedling emergence were observed at PEC and 5PEC in the ES and Italy (IT) mixtures and at 5PEC in the SI mixture in wheat (Figure 2, see online supplementary material Figure S2). In lettuce, significant reductions in seedling emergence were observed in the CZ, NL, and AR mixtures at PEC and 5PEC (Figure 2).

Shoot biomass of wheat plants decreased significantly at all concentrations in the ES and SI mixtures and at PEC and 5PEC in the AR, CZ, IT, and NL mixtures (Figure 2, see online supplementary material Figure S2). In relation to solvent control, wheat biomass showed a significant reduction of 57.1% to 62.8% at MEC, 12.3% to 48.0% at PEC, and 0% to 37.0% at 5PEC. In lettuce, shoot biomass decreased significantly at all concentrations in the AR, NL, and SI mixtures; at PEC in the CZ and ES mixtures; and at 5PEC in the DK and IT mixtures (Figure 2). In relation to solvent control, lettuce biomass exhibited a significant decline of 57.1% to 62.8% at MEC, 12.3% to 48.0% at PEC, and 0% to 37.0% at 5PEC.

No effects on seedling emergence or growth were observed in either species for the France (FR), CH, HR, and PT mixtures and in wheat for the DK mixture.

Principal component analysis (see online supplementary material Figure S3) summarized multivariate mixture responses,

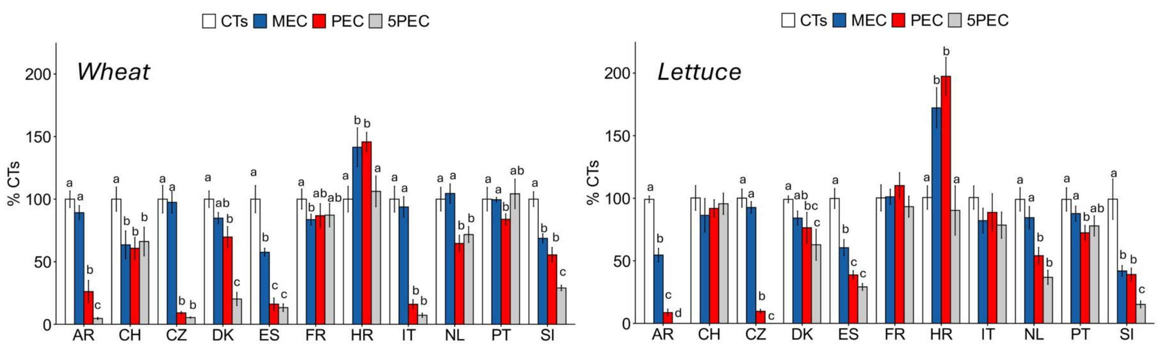

Figure 1 Root growth (mean ± *SD*) expressed as percentage of solvent control in wheat (*Triticum aestivum*) and lettuce (*Lactuca sativa*) exposed to the MEC, PEC, and 5PEC, all expressed as nominal concentrations. Different letters denote significant differences (Tukey-type post hoc test, $p < 0.05$). CT = solvent control; MEC = median measured environmental concentration; PEC = predicted environmental concentration; 5PEC = 5-times PEC; AR = Argentina; CH = Switzerland; CZ = Czech Republic; DK = Denmark; ES = Spain; FR = France; HR = Croatia; IT = Italy; NL = the Netherlands; PT = Portugal; SI = Slovenia.

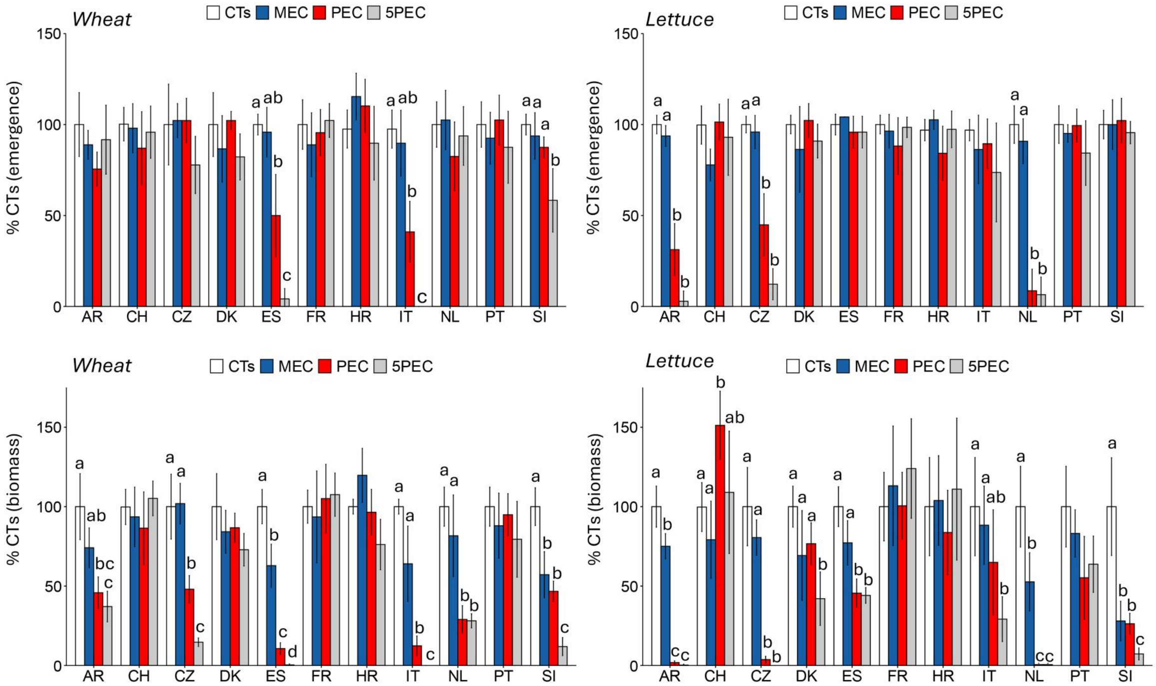

Figure 2 Seedling emergence (top) and shoot biomass (bottom; mean ± *SD*) expressed as percentage of solvent control in wheat (*Triticum aestivum*) and lettuce (*Lactuca sativa*) exposed to the median measured environmental concentration (MEC), predicted environmental concentration (PEC), and 5-times PEC (5PEC), all expressed as nominal concentrations. Different letters denote significant differences (Tukey-type post hoc test, $p < 0.05$). CT = solvent control; AR = Argentina; CH = Switzerland; CZ = Czech Republic; DK = Denmark; ES = Spain; FR = France; HR = Croatia; IT = Italy; NL = the Netherlands; PT = Portugal; SI = Slovenia.

with PC1 (58%) and PC2 (19%) explaining 77.7% of the variability, revealing two distinct clusters (CZ, AR, NL vs. ES, IT, SI), while PC1 reflected positive contributions from all endpoints and PC2 distinguished wheat from lettuce (see online supplementary material Table S2).

Discussion

Effects of pesticide mixture on terrestrial plants

The toxicity of 11 pesticide mixtures was assessed in standard ecotoxicity tests using 2 plant species. The mixtures were selected by pesticide residues found in agricultural soils, and the concentrations were chosen to include environmentally relevant concentrations, such as MEC and PEC. Overall, our results confirm previously reported findings that responses are species and endpoint specific (Serra et al., 2019).

The root growth test was found to be the most sensitive endpoint, with significant effects at MEC observed in four mixtures in each species. In wheat, effects at MEC occurred in CH, ES, and FR mixtures, which were the only ones containing difenoconazole and AMPA in their composition. Although difenoconazole reduced wheat root length in hydroponic exposure (Liu et al., 2021), effects of the IT mixture containing difenoconazole were observed only at 5PEC. This indicates that difenoconazole alone was unlikely to explain the observed responses at lower, MEC concentrations. AMPA alone was also not likely to cause toxicity at the exposure concentration (i.e., MEC), since the only record of AMPA toxicity to wheat in a soil exposure occurred at concentrations 2 times higher than its concentration at 5PEC (Blackshaw & Harker, 2016). Because individual pesticides were not expected to be toxic at the MEC, these effects likely reflected the combined influence of multiple pesticides. Moreover, it is interesting to note that the FR and CH mixtures did not affect any other endpoint. These two mixtures contained the highest number of fungicides (three in each mixture). While fungicides are known to disrupt photosynthesis through various physiological processes in terrestrial plants (Petit et al., 2012), their contribution here remains unclear.

In lettuce, root growth was reduced in four mixtures (AR, DK, ES, and SI), while shoot biomass was affected in three mixtures (AR, NL, and SI) at MEC. At PEC and 5PEC, CZ, IT, and PT mixtures also affected one of the endpoints. No clear relationship emerged between mixture composition and the observed patterns of toxicity (Table 1, see online supplementary material Table S3). Photosynthesis II inhibitors were present only in NL and SI mixtures (metobromuron and metribuzin in the NL mixture and terbuthylazine in the SI mixture), while AR and DK mixtures contained diflufenican, a carotenoid biosynthesis inhibitor, which inhibits chlorophyll production. These findings indicate that the overall toxicity could not be explained by individual herbicides or their modes of action alone. Therefore, the specificity of responses suggests that combined mixture effects shaped plant sensitivity.

According to the PCA results (see online supplementary material Figure S3), CZ, AR, and NL mixtures were clustered, indicating a comparable impact on the study endpoints. The position of this cluster also indicated a stronger effect on lettuce plants.

These mixtures had in common the herbicides AMPA and diflufenican and no fungicide in their composition. The role of AMPA in observed toxicity was probably low, as previously discussed. Diflufenican is a carotenoid biosynthesis inhibitor, normally applied in winter cereals among other crops (Pesticide Properties Database, 2025). The lowest reported half-maximal effective concentration of diflufenican for effects on plant fresh weight (0.229 mg kg^{-1}; European Food Safety Authority, 2007) is comparable to the PEC value used in the experiments (0.25 mg kg^{-1}), suggesting that this compound may have contributed to the observed toxicity. Moreover, CZ and AR mixtures contained synthetic auxins (mecrocop-P and 2,4-dichlorophenoxyacetic acid [2,4-D], respectively), which are growth regulators that can be absorbed by the roots and interfere with cell formation. Triques et al. (2021) observed effects of 2,4-D on growth and emergence in *Allium cepa* and *Raphanus sativus* plants at concentrations as low as 1.175 mg.kg^{-1}, with the dicotyledonous species being more sensitive, in accordance with the findings of the present study.

The second cluster comprised ES and IT mixtures. These mixtures had in common the herbicide propyzamide and the fungicide difenoconazole. Propyzamide is a microtubule assembly inhibitor affecting cell division. Regarding the fungicide difenoconazole, Pitombeira et al. (2020) observed that monocotyledonous species were more sensitive than dicotyledonous species. Both ES and IT had similar effects and were the only mixtures affecting seedling emergence and biomass in wheat at the PEC treatment.

The SI mixture, located between the two clusters in the PCA plot, was one of the most toxic mixtures, affecting root or seedling growth endpoints for both species at MEC or PEC treatments. However, this mixture caused no effect on seedling emergence. Although the herbicides in its composition—namely, *S*-metolachlor, terbuthylazine, and thiencarbazone-methyl—interfere with early plant development, their mode of action does not directly target emergence and may require a certain activation time before phytotoxic effects become apparent, which may explain this pattern (see online supplementary material Table S3). For example, thiencarbazone-methyl is known to produce phytotoxic effects only several days after application.

In the HR mixture, seedling and root growth were higher at MEC and PEC in comparison with controls in both species. This pattern is consistent with a hormetic-type response, in which low doses of a stressor stimulate growth rather than inhibit it. The HR mixture contained only glyphosate as herbicides, its metabolite AMPA, the fungicides trifloxystrobin and tebuconazole, and the insecticide deltamethrin. A similar stimulatory effect was observed for shoot biomass in lettuce at PEC treatment in the CH mixture. In this mixture, three fungicides, one insecticide, and AMPA were present, therefore making it difficult to identify which pesticide or pesticides may have contributed to the stimulatory effect. Notably, both mixtures showing stimulatory effects contained tebuconazole and AMPA, although no studies on hormesis effects of these substances are available. Glyphosate, however, has been reported to cause hormesis, but the mechanisms remain unclear (Belz & Duke, 2014). In some cases, it appears to be related to its mode of action, such as partial inhibition of the 5-enolpyruvylshikimate-3-phosphate synthase enzyme, which reduces lignin biosynthesis, increases cell wall elasticity, and allows enhanced cell elongation. Others appear unrelated to its toxic action, such as increased photosynthesis in plants exposed to glyphosate under certain light and temperature conditions. Nevertheless, other mixtures containing glyphosate did not show stimulatory effects. Taken together, these findings suggest that growth stimulation at low doses may occur for certain pesticide combinations, but confirmation requires appropriate dose–response experiments.

Assessing realistic exposure scenarios is critical for evaluating environmental risk of pesticides. Although our study emphasizes soil residues, drift is a major exposure route to NTTPs. Still both pathways are important, as drift exposure represents immediate deposition on the plants following application, and soil residues can potentially cause long-term exposure risks. Moreover, physicochemical properties of pesticides (e.g., persistence, solubility, volatility) determine the relative importance of each pathway. For example, pesticides with a high octanol–water partition coefficient strongly bind to soil particles, reducing drift exposure but increasing soil residue accumulation. Considering drift and soil residue exposure as complementary pathways is therefore essential for assessing the environmental risk of complex pesticide mixtures.

Risk assessment and study limitations

Currently, spray drift is the primary route of exposure considered in the European Union risk assessment for NTTPs. For this exposure route, single-substance toxicity tests are generally deemed sufficient, since pesticides are normally not applied simultaneously and exposure can be reduced by implementing buffer zones. However, this approach overlooks pesticide residues in the soil matrix as an additional exposure route. In the present work, considering both species, significant effects in root growth or seedling biomass were observed at the MEC treatment in 7 mixtures (AR, CH, DK, ES, FR, NL, and SI), while at PEC, significant effects were observed in 10 mixtures (all except HR). It is worth reminding here that (a) the MEC treatments corresponded to levels found in real agricultural soils (Knuth et al., 2024) and (b) the PEC treatments corresponded to the levels that are considered safe for approved pesticides based on their regulatory assessment (if the PEC concentration was found unsafe, the pesticide would not be among the approved ones; European Commission, 2002). While we acknowledge that effects were not observed for all mixtures at the MEC exposure, our results provide evidence that multiple pesticide residues in soil can adversely affect terrestrial crops. This highlights the need to refine current risk assessment frameworks to better reflect real-world pesticide exposure scenarios and to consider the limitations of extrapolating from crop species to wild plants.

In European Union risk assessment, seedling emergence and growth and vegetative vigor tests are normally conducted with 6 to 10 species. In contrast, our study was limited to 2 species and focused solely on seedling emergence and growth. The use of crop species (radish and cress) does not fully represent wild plant responses. Despite this narrower scope, the root growth test proved to be highly sensitive for both species. Root test is faster and requires fewer resources than other tests. However, because the toxicity of some herbicides manifests only a few days after application, the short duration of the root growth test here in regulatory contexts may not capture their full toxic effects. Longer exposures could also inform on temporary effects and plant recovery (Vercampt et al., 2016). Besides this short-term aspect, a few other limitations were related to experimental design and procedures. First, only mixtures were tested (i.e., no single exposure testing), so deviations from additive effects cannot be confirmed. This test design, similar to a whole-mixture approach, was chosen to evaluate a large number of mixtures while minimizing the number of experiments (Tourinho, Hochmanová, Kukučka, Jegede, et al., 2025). Second, all experiments were conducted in a single soil type, while it is known that pesticide bioavailability and toxicity vary substantially across soil types. Third, no analytical verification of residue concentrations in the soil after spiking was performed, and all reported concentrations are nominal values. Therefore, there was no verification of how closely related the tested concentrations are to the environmental concentrations. Finally, no mechanistic analyses (e.g., chlorophyll fluorescence parameters) were included in this study. Yet, our findings provide a basis for future work to investigate the physiological mechanisms underlying pesticide-induced inhibition in plants. Such mechanistic studies would be particularly valuable for understanding why certain mixtures showed strong effects and how different pesticide combinations may exert toxicity through distinct pathways.

Conclusions

In this study, the toxic effects of realistic pesticide mixtures at environmentally relevant concentrations, based on nominal concentrations, were observed in two terrestrial plants. The responses, species and endpoint specific, may relate to the mode of action of the herbicides and the period of activation of herbicides that are designed to produce effects on weeds at a certain period after their application on crops. Moreover, effects were observed in mixtures with predominance of fungicides, indicating that all classes of pesticides can affect nontarget plants, especially when in mixtures.

Terrestrial plants exhibited high sensitivity to pesticide mixture exposure, showing significant impacts on seedling biomass and root growth at MEC levels, although these effects were not consistently observed across all experiments. The occurrence of effects at MEC highlights that the current real presence of multiple pesticide residues in soil represents an important exposure pathway that should be carefully considered in environmental risk assessments.

Given that current European risk assessment frameworks primarily focus on spray drift and single-compound exposures, our results highlight the need to consider the mixture effects of pesticide residues in the evaluation of pesticide risks to NTTPs. Considering the limitations of the present work, future studies should include longer-term experiments and test single-substance exposures to address interactive effects and potential recovery processes.

Supplementary material

Supplementary material is available online at *Environmental Toxicology and Chemistry.*

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author contributions

Paula S. Tourinho (Conceptualization, Formal analysis, Investigation, Visualization, Writing—original draft), Vera Silva (Funding acquisition, Project administration, Writing—review & editing), Paula Harkes (Funding acquisition, Project administration, Writing—review & editing), Trine Nørgaard (Writing—review & editing), Virginia Aparicio (Writing—review & editing), and Jakub Hofman (Conceptualization, Supervision, Writing—review & editing)

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Conflicts of interest

None declared.

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