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Microplastics and PFAS as ubiquitous pollutants affect potencies of highly toxic chemicals in mixtures

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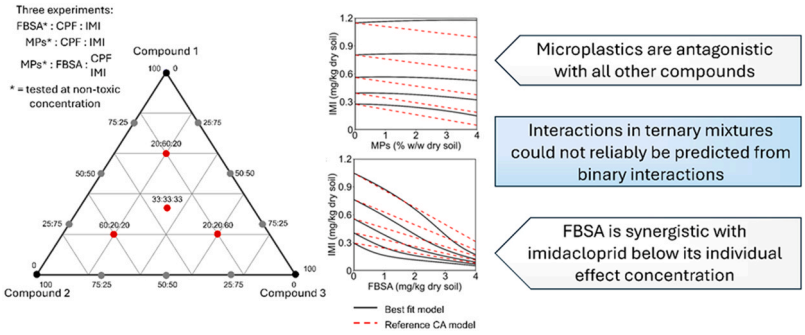
HIGHLIGHTS

- First ternary mixture tests with microplastics, PFAS and insecticides on springtails.
- Less effects of FBSA and insecticides in the presence of microplastics.
- FBSA at toxic levels decreased the effects of the other compounds.
- FBSA at non-toxic levels enhanced the effect of imidacloprid.
- Ternary toxicity interactions cannot be reliably predicted from binary interactions.

GRAPHICAL ABSTRACT



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ABSTRACT

Pollutants usually occur as mixtures in the environment, where they affect each other's toxicity even at non-toxic concentrations. Despite the environmental relevance of interactions in complex mixtures, they remain severely understudied. Therefore, this study aimed to assess how PFAS and microplastics (MPs) as ubiquitous pollutants at (non-)toxic concentrations affect the potencies of highly toxic chemicals in mixtures. Experiments were performed to assess the toxicity of perfluorobutane sulfonamide (FBSA), linear low-density polyethylene MPs (0–50 μm), chlorpyrifos and imidacloprid to the reproduction of the springtail *Folsomia candida* in Lufa 2.2 soil, as single compounds, binary and ternary mixtures. FBSA at non-toxic concentrations showed dose-dependent synergism with imidacloprid, but antagonism with chlorpyrifos. Synergism only occurred in the ternary mixtures dominated by FBSA at non-toxic concentrations and imidacloprid. MPs and toxic concentrations of FBSA were antagonistic in all mixtures. In conclusion, MPs and FBSA affected insecticide toxicity, and assuming concentration addition for the hazard assessment of ternary mixtures is generally precautionary. Ternary interactions could not be reliably predicted from binary interactions, and ratio-specific interactions may be

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overlooked. This emphasizes the need to include experimental mixture toxicity data for a realistic risk assessment of complex chemical mixtures.

1. Introduction

The number of novel chemicals has increased exponentially over time, with the chemical abstract service (CAS) registry numbers rising from 20 million in 2000 to approximately 219 million today [8,11]. Many of these chemicals are widely used in industrial and consumer products, and have consequently entered the environment through various pathways, leading to continuous exposure of organisms to complex mixtures of contaminants [60]. However, to date, only a relatively small fraction of CAS-registered chemicals has been analyzed in environmental media and studied for their (eco)toxicological effects [13,33], with even far less knowledge available regarding the effects of complex mixtures of chemicals, hampering comprehensive understanding and thereby a realistic environmental risk assessment of chemical mixtures.

In recent decades, microplastics (MPs) and per- and polyfluoroalkyl substances (PFAS) have become contaminants of major global concern due to their environmental ubiquity and potential hazard to environmental health [21,56]. Yet, MP and PFAS concentrations in the environment remain poorly quantified, especially in more complex matrices like soil. For MPs, this may be due to a lack of proper quantification and characterization techniques, especially for particles smaller than 5–10 μm [22]. Despite these analytical uncertainties, environmental concentrations of over 1 % (w/w) have been reported [69,6]. PFAS usually occur in the environment as structurally diverse mixtures, of which detection and quantification are also still constrained by analytical standards and techniques [21,39], albeit this is gradually improving [44]. This resulted in a reported average sum PFAS (44 compounds) concentration of 0.183 mg/kg dry soil [20]. Because toxicity of MPs is directly related to their morphology (e.g., shape and size), and that of PFAS to their structural properties [21,4], the lack of detailed analytical techniques to define MP and PFAS exposures in the environment is hindering their hazard and risk assessment. Despite a growing body of literature in the past years, the toxicity of MPs and PFAS to soil organisms remains poorly understood [21,6]. Moreover, the available knowledge on the (eco)toxicity of PFAS is strongly biased towards a limited number of long-chain legacy PFAS due to regulatory needs, leaving the vast majority of (emerging) PFAS, like short-chain perfluoroalkyl sulfonamides (PFASAs), understudied and therefore unregulated [14,21,5].

On top of the substantial knowledge gaps in determining the effects of MPs and PFAS on environmental health, these ubiquitous contaminants may also interact with each other as well as with other co-occurring chemicals in the environment, further complicating risk assessment. MPs are often assumed to act as vectors for other contaminants, facilitating their transport into organisms depending on the lipophilicity of the associated chemicals (e.g. [18,26,37,42,59]), also known as the Trojan Horse mechanism. This has previously been documented for aquatic matrices and is assumed to take place in soil as well, although empirical evidence is lacking [1,34,53,66]. PFAS may interact with various transporters, proteins, receptors and cell membranes due to their amphiphilic properties, thereby potentially altering the bioavailability and toxicity of other chemicals [52,67]. Some PFAS have been reported to affect the immune system and cytochrome P-450 (CYP-450) enzymes, which may therefore cause synergism in complex mixtures of contaminants due to reduced detoxification, or antagonism due to reduced bioactivation of other contaminants [40,63,10]. Moreover, both MPs and PFAS can directly influence the surrounding soil matrix by altering pH, water holding capacity or structure of the organic matter, potentially affecting the sorption and bioavailability of other contaminants [24,30,31,51,57]. Hence, the argument that the toxicity of MPs and PFAS occurs at concentrations higher than those commonly found in the environment [7,50,61] ignores their interactions with other chemicals that may still occur at non-toxic concentrations. This may be the case for the entire plethora of compounds present in the environment at relatively low concentrations, which remains virtually unknown and thus needs urgent clarification.

The aim of the present study was therefore to assess how short-chain PFAS and MPs as ubiquitous pollutants affect potencies of highly toxic chemicals in mixtures. To unravel these potential interactions, we performed ternary mixture toxicity experiments, examining whether PFAS and MPs at (non-)toxic concentrations would influence the toxicity of insecticides, and whether the amphiphilic PFAS would alter the potential interaction between MPs and a hydrophilic and a lipophilic insecticide. To explore if the effects of the ternary mixtures could be predicted from those of the less complex binary mixtures, the toxicity of the single chemicals and all binary combinations was also assessed simultaneously with the corresponding ternary mixture.

Linear low-density polyethylene (LLDPE) was selected as model MP and perfluorobutane sulfonamide (FBSA) as model short-chain PFAS, since it is an emerging PFAS of concern [65,64]. The two selected insecticides were the hydrophilic imidacloprid (log K_{ow} = 0.57), an acetylcholine receptor agonist, and the lipophilic chlorpyrifos (log K_{ow} = 4.96), an acetylcholinesterase inhibitor. Imidacloprid and chlorpyrifos were selected also because of their widespread use and detection in soils across the globe, with concentrations reaching up to 1.1 and 4 mg/kg dry soil, respectively [16,19,3,49,70]. The springtail *Folsomia candida* was selected because it is a standard soil test organism, for which standardized test guidelines are available [35].

2. Materials and methods

2.1. Outline of the study

Three mixture toxicity experiments were conducted in a full factorial design (Figure S1). The chemical components and the objective(s) of each experiment are summarized in Table 1. Concentration ranges of the different components in the mixtures were chosen to unravel their potential interactions in the mixtures. Consequently, the selected concentration ranges for insecticides and MPs included environmentally relevant levels, but were higher for FBSA [29,3,69,70]. The toxicity tests were performed according to OECD guideline 232, with modifications [35]. The reproduction of adult springtails after 21 days of exposure was taken as the sub-lethal endpoint.

Table 1
Chemicals tested and objective(s) of each mixture toxicity experiment performed in this study, using the springtail *Folsomia candida* exposed for 21 days in Lufa 2.2 soil. Asterisks indicate chemicals tested at non-toxic concentrations. FBSA = perfluorobutane sulfonamide; CPF = chlorpyrifos; IMI = imidacloprid; MPs = linear low-density polyethylene microplastics.

Experiment No.	Chemicals	Objective(s)
Experiment 1	FBSA* CPF IMI	To assess the effects of FBSA at non-toxic concentrations on the toxicity of two insecticides and their mixture.
Experiment 2	MPs* CPF IMI	To assess the effects of MPs at non-toxic concentrations on the toxicity of two insecticides and their mixture.
Experiment 3	MPs* FBSA CPF IMI	To assess the effects of MPs on the toxicity of PFAS and two insecticides. To assess the effect of the amphiphilic PFAS on the interaction between MPs and two insecticides differing in lipophilicity.

2.2. Test organisms and culture conditions

Folsomia candida (Willem, 1902) were obtained from cultures at the Vrije Universiteit Amsterdam, kept in a climate room at 16°C, with a 16:8 h light:dark photoperiod and a relative humidity of 75 %. Cultures were kept on moist plaster of Paris with a 10:1 ratio of activated charcoal, and watered and fed weekly with dried baker's yeast (AB|Mauri Netherlands, Dordrecht, The Netherlands). Age synchronized animals, 20–22 days old, were used for all experiments. Adult animals were used to increase the possible ingestion of the MPs, as their mouth parts are substantially bigger than those of juveniles. Age-synchronizations were performed by allowing adults from the cultures to oviposit for 48 h on moist plaster of Paris. The synchronizations were kept in a climate room under the same conditions except for a temperature of 20°C.

2.3. Test soil

Natural standard Lufa 2.2 soil (Lufa Speyer, Germany) from a single batch was used. Upon arrival, the soil was dried at 40°C for 48 h to prevent mold formation. According to the supplier, the sandy loam soil contained 1.66 ± 0.60 % organic carbon, and had a water holding capacity (WHC) of 44.2 ± 6.0 % and a pH (0.01 M $CaCl_2$) of 5.5 ± 0.1.

2.4. Test compounds and soil preparation

Microplastics were produced, the microplastic test material was obtained from NanoFract (Germany). To include particles that are environmentally relevant for the soil ecosystem, a polymer that is often found in (agricultural) soil was used [69]. Linear low density polyethylene (LLDPE) also proved to be more easily micronized down to the required particle size, compared to for example LDPE, ensuring sufficient ingestion possibility for *F. candida*. LLDPE was micronized until 90 % of the particles were between 10 and 50 μm in size. A small percentage of particles were larger than this, typically in the size range of 100–200 μm representing unmilled residues that could not be completely separated from the milled material (see Figure S2a & Figure S2b). The particles were characterised by morphology, size, and polymeric composition (see Supplementary Information). The majority of the particles were very fine, resembling elongated, flattened filaments produced through the micronization process which form weak agglomerations when mixed (Figure S2a). The LLDPE composition of the material was confirmed with attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy, by the presence of a small peak at 1377 cm^{-1} in addition to other characteristic peaks of polyethylene (Figure S2c)[28].

Perfluorobutane sulfonamide (FBSA; CAS no. 30334–69–1; 97 % purity) was purchased from Apollo Scientific (Bredbury, United Kingdom), and imidacloprid (PESTANAL, ≥98 % purity) and chlorpyrifos (PESTANAL, ≥98 % purity) from Merck Germany. Due to its high lipophilicity (Log K_{ow}= 4.96), chlorpyrifos was added to the soil using acetone (99.8 % purity, VWR chemicals, The Netherlands) as a carrier solvent. Specifically, 30 g of dry soil (20 % of the total weight of dry soil needed for each treatment) was put in a glass jar (78 mm × 63 mm; diameter × height), followed by the addition of chlorpyrifos dissolved in 25 mL of acetone to just submerge the soil and to reach the required nominal chlorpyrifos concentration in the entire amount of dry soil (150 g) per treatment. To exclude possible effects of the carrier solvent as a confounding factor, all the other soils than the negative controls were treated with only acetone as well, but without chlorpyrifos. The jars containing acetone-treated soil were closed and left to equilibrate for 24 h, after which the lids were opened, and the soil was left to dry in a fume hood under constant stirring. In the case of chlorpyrifos, this prevented the formation of a crust of the compound on top of the treated soil. When the soil was almost completely dry, the jars were left open in the fume hood for 24 h to ensure all acetone had evaporated. The acetone-treated soil was then mixed with the remaining (80 %) untreated dry soil to obtain the required batches of soil for the addition of the other compounds, and the preparation of the solvent controls. The MPs were mixed in with the mixtures of untreated (adding up to 80 %) and the acetone-treated (20 %) portions of soil, following the recommendations of Jemec Kokalj et al. [25]. In this way it was prevented that the MPs would come into contact with and be damaged by acetone and damage to the structure of the soil organic matter fraction by the acetone was limited. Demineralized water was then mixed in with the soil to reach 50 % WHC. When a specific treatment included FBSA and/or imidacloprid, these compounds were pipetted into the demineralized water from a stock solution before being added to the soil.

2.5. Experimental setup

The concentration ranges of the different compounds varied depending on whether the compounds were tested individually, or in binary or ternary mixtures and on the dose ratios of the compounds in the mixtures. To allow for composing all these different mixtures, standard exposure levels (SELs) were defined for all compounds. For the MPs, no effect concentrations were available, since in previous experiments hardly any effects were observed at MP concentrations up to 5 % [57]. Therefore, 1 % (w/w dry soil) was taken as one SEL. For the non-toxic concentrations of FBSA (in Experiment 1), 1 mg/kg dry soil was chosen as one SEL, ensuring that even the highest test concentration was below the NOEC, so that FBSA alone would have no effect on springtail reproduction. For the toxic concentrations of FBSA (in Experiment 3) and the two insecticides in all experiments, one SEL corresponded to one toxic unit (TU), equal to EC_{50} values taken from range finding experiments (Text S1, Figure S3) or from Selonen et al. [46] and de Lima e Silva et al. [12], respectively. One TU was 5.58 mg/kg dry soil for FBSA, 0.1 mg/kg dry soil for chlorpyrifos and 0.3 mg/kg dry soil for imidacloprid, respectively. For each treatment, both for single chemicals and mixtures, concentrations of 0.25, 0.5, 1, 2, and 4 SELs were tested. In addition to the negative control, a solvent control was incorporated as well, in which animals were exposed in soil of which 20 % was treated with acetone, like the other treatments, but without toxicants (Figure S1 and Table S1).

The mixtures were composed according to Cedergreen et al. [9], with binary mixtures of the compounds tested at concentration ratios of 25:75, 50:50 and 75:25 and ternary mixtures at ratios of 20:20:60, 20:60:20, 60:20:20 and 33:33:33. The single compounds were tested simultaneously with the mixtures to ensure proper comparison and analysis of the interactions in the mixtures.

There were five replicates per treatment. An individual replicate consisted of a glass jar (100 mL) filled with 30 ± 0.5 g of moist soil, to which ten adult springtails randomly selected from the synchronizations were added along with 5 mg dried baker's yeast as food. To replenish evaporated water, jars were restored to their original weight by adding demineralized water every week, and 5 mg dried baker's yeast was added weekly as food.

After the end of the 21-day tests, 100 mL of water was used to rinse the test soils and animals from the test jar into a beaker. The suspension was then gently stirred causing the animals to float to the surface, after which pictures of the water surface were taken using a Sony ILCE-6500 digital camera. The animals floating on the surface were counted in Fiji ImageJ (version 1.8.0_172). For treatments containing over 1 % (w/w dry soil) of MPs, the soil suspensions were poured into larger beakers using 600 mL of water, to increase the surface area of the water surface. This was done to avoid the formation of a thick layer of MPs at the surface, preventing the animals from floating to the surface. Pictures were taken both with and without the addition of Indian ink, to increase contrast.

2.6. Soil pH measurements and chemical analysis

After mixing the compounds into the soil, two 6 ± 0.05 g soil samples

were taken from the soil batches of 0.25, 1 and 4 SELs from all treatments for pH measurements, as well as 25 g of soil from the same batches for chemical analysis. Soil pH was measured by adding 24 mL of 0.01 M $CaCl_2$ to 6 g of moist soil, approximately the equivalent of 5 g of dry soil. The suspension was shaken for 2 h on a shaker at 200 rpm and left overnight to settle. pH was measured in the supernatant using a WTW pH 7710 digital pH meter.

Chemical analysis of the FBSA concentrations in soil samples was performed in duplicate. The extraction, clean-up and analysis of soil samples were based on methods previously described by Xie et al. [64]. Quantitative analysis of FBSA was performed by a Nexera UHPLC system (Shimadzu, Kyoto, Japan), coupled with a Bruker MaXis 4 G high-resolution q-TOF-HRMS with an Ion Booster Electro Spry Ionization (IB-ESI) source, operated in negative mode. Chromatographic separation of FBSA was performed on an Acquity UPLC CSH C18 column (130 Å, 2.1 × 150 mm, 1.7 µm). The limit of quantification (LOQ) was 0.153 µg/kg dry soil.

Chemical analysis of chlorpyrifos and imidacloprid was performed by Normec Groen Agro Control (Delfgauw, The Netherlands), employing certified (ISO17025; EU2017/625) liquid chromatography-tandem mass spectrometry methods, with a reporting LOQ of 0.01 mg/kg dry soil. As the first round of chemical analysis yielded recoveries lower than expected for chlorpyrifos, insecticide concentrations were checked again in the lab of Wetterskip Fryslân to ensure they were correct. Chlorpyrifos and imidacloprid were analyzed by using gas chromatography-tandem mass spectrometry and liquid chromatography-tandem mass spectrometry, with limits of detection of 0.6 µg/kg dry soil and 0.3 µg/kg dry soil, respectively.

2.7. Mixture toxicity modelling and statistical analysis

Logistic dose-response curves of the single compounds were constructed and analysed in R-Studio (R-Core Team, v4.3.2) using the R package *'drc'* (v3.0.1[43]).

The interactions between the two compounds in the binary mixtures were descriptively modelled and fitted using the MIXTOX Tool by Jonker et al. [27]. This tool uses a three-parameter dose-response model with the parameters Y_{max} (maximum asymptote of the dose response curve), *Slope* and EC_{50} (50 % effect concentration). In the binary interaction models, these parameters were first estimated by fitting the model to the data of the single compounds within the binary mixture. These parameters were then fixed and only the interaction parameters a for antagonism/synergism and b_{DR} (note: this is b_i as defined by Jonker et al. [27]) and b_{DL} for dose-ratio and dose-level dependent deviations from additivity, respectively were fitted for the binary mixtures, using the single and binary mixture data. Since no EC_{50} value was available for the MPs, the EC_{50} value used in the mixture toxicity modelling was fixed at three times the NOEC (4 % w/w dry soil), resulting in a modelling EC_{50} of 12 % MPs (w/w dry soil). As a model sensitivity check and to ensure that the conclusions drawn from the analyses are not dependent on this EC_{50} assumption, the full analyses were also performed using assumed EC_{50} values of 6 % and 24 % (Text S2, Figures S4 and S5). Isoboles were plotted for binary mixtures to visualise the interactions between the compounds, using the package *'ggplot2'* (v3.4.4; [62]).

Ternary interactions were modelled in an Excel model based on the MIXTOX Tool, modified to analyse interactions between three compounds [9]. This tool uses the same three-parameter dose-response model with the parameters Y_{max}, *Slope*, and EC_{50} as the MIXTOX Tool. In the ternary interaction model analysis, these parameters were first estimated by fitting the model to the data of the single compounds within the mixture. Then the binary mixture interaction parameters *a1* (compounds 1 vs. 2), *a2* (2 vs 3) and *a3* (1 vs 3) were estimated, using the single and binary mixture data, but without changing the Y_{max}, *Slope* and EC_{50} values. Next, also the *a1*, *a2* and *a3* parameters were fixed and the *a4*, the additional ternary interaction parameter, was fitted using all the single, binary and ternary mixture data. Finally, the ternary mixture interaction parameters (*a4*) were also estimated for each individual ternary mixture ratio by fitting the model using the single, binary and the data of each single ternary ratio data individually (i.e. excluding data from the other ternary mixtures from the analysis), to analyze and compare the ternary interactions between the different concentration ratios of the compounds in the ternary mixture. This allows for the analysis and identification of additional ratio-specific ternary interactions that are otherwise not visible, as the overall hefty antagonistic interactions for certain concentration ratios will force the fitting of the global *a4* to "average out" a slight synergism at the remaining ratios of the three compounds in the mixture, or the other way around. This may lead to the conclusion of overall antagonism, even when interactions at some concentration ratios are in fact synergistic. This interaction may still be relevant when analyzing the ternary mixture in terms of deviations from concentration addition. The ternary isoplanes (three-dimensional isobole plane of the four-dimensional dose response curves, showing the EC_{50} plotted as a function of the concentration of the three compounds in the mixture) were plotted using the R package *'plotly'* (v4.10.4; [48]).

Significance of deviations from the concentration addition model (antagonism/synergism, dose-level or dose-ratio dependent deviations), for both the binary and the ternary mixtures, were determined using maximum likelihood analysis, as described in Jonker et al. [27].

3. Results

3.1. Test validity

Overall performance of the springtails met the validity criteria of OECD guideline 232 for assessing the toxicity of chemicals to collembolan reproduction [35]. Adult control survival exceeded 80 %, reproductive output was at least 100 juveniles per jar, and the coefficient of variance was below 30 %. In Experiment 1, reproduction in controls from the second batch (567 ± 50.1; mean ± SD, n = 10) was significantly lower (Student's T-test, $p < 0.001$) than that from the first batch (813 ± 72.8; n = 9). To allow data to be analyzed together, the number of juveniles in the replicates started with animals from the second batch were multiplied by the factor difference (1.43) between the two batches. In all experiments, control and solvent control survival and reproduction did not differ significantly (Student *t*-test, $p > 0.05$). Soil pH showed only small variations across treatments, and no chemical concentration-related trends were observed (Table S1).

3.2. Measured concentrations of test compounds

Measured concentrations of test compounds are reported in Table S2. Average recovery of FBSA was 116 % ± 11.9 % (mean ± SD, n = 24). FBSA was not detected in the procedural blanks. According to the first lab, average recovery of chlorpyrifos and imidacloprid was 62.1 % ± 16.5 % and 70.4 % ± 21.6 % (mean ± SD, n = 28 and 31), respectively. According to the second lab, the average recovery of chlorpyrifos and imidacloprid was 17 % ± 3 % and 80 % ± 8 % (mean ± SD, n = 6), respectively. The lower than expected recovery for chlorpyrifos measurement at the first lab could have resulted from the possible degradation of chlorpyrifos between end of experiments and analysis, with even further degradation prior to analysis at the second lab. As effect concentrations of chlorpyrifos were well in line with earlier observations, and also because of the good recoveries for FBSA and imidacloprid, all data analysis used nominal exposure concentrations. As each experiment made use of a single stock solution for each of the individual compounds, and simultaneously tested the single chemicals and the mixtures, the poor recovery would not affect the interpretation of the mixture interactions.

3.3. Adult survival

Since the test concentrations were based on the sublethal endpoint reproduction, most compounds did not cause more than 50 % springtail mortality in any of the experiments. It was therefore not possible to plot proper dose-response curves for the survival effects of most compounds, nor to evaluate the interactions between two or three compounds in the mixtures regarding the endpoint survival. Some interactions were observed, like a strong antagonistic interaction between FBSA and chlorpyrifos effects on springtail survival (for actual survival data and visualization of the interaction, see the Zenodo link in the data availability statement). However, the evaluation of survival was not the aim of the study and is therefore not further analyzed as such.

3.4. Toxicity of single compounds

As expected, FBSA at non-toxic concentrations in Experiment 1 and MPs at non-toxic concentrations in Experiments 2 and 3 did not cause any effects on springtail reproduction. FBSA at toxic concentrations and the two insecticides indeed proved toxic to the reproduction of *F. candida* at the chosen concentration range, leading to > 50 % effect in all tests. The dose-response curves fitted for the single compounds are shown in Fig. 1, and the corresponding model parameters in Table 2. The EC_{50} for FBSA in Experiment 3 was calculated to be 5.17 mg/kg dry soil. Chlorpyrifos and imidacloprid showed some variations in EC_{50} values across the three experiments (Table 2).

3.5. Toxicity of binary mixtures

Significant interactions between the two compounds were observed in all binary mixtures (Table 3, Fig. 2). Synergistic interactions were only observed for the mixture of non-toxic FBSA concentrations and imidacloprid (Fig. 2). These interactions were dose-level dependent, indicating that the magnitude of the synergism depended on the FBSA concentration in the mixture (Fig. 3). The degree of synergism was quite large, with a three times lower EC_{50} for imidacloprid at the ratio of 25:75

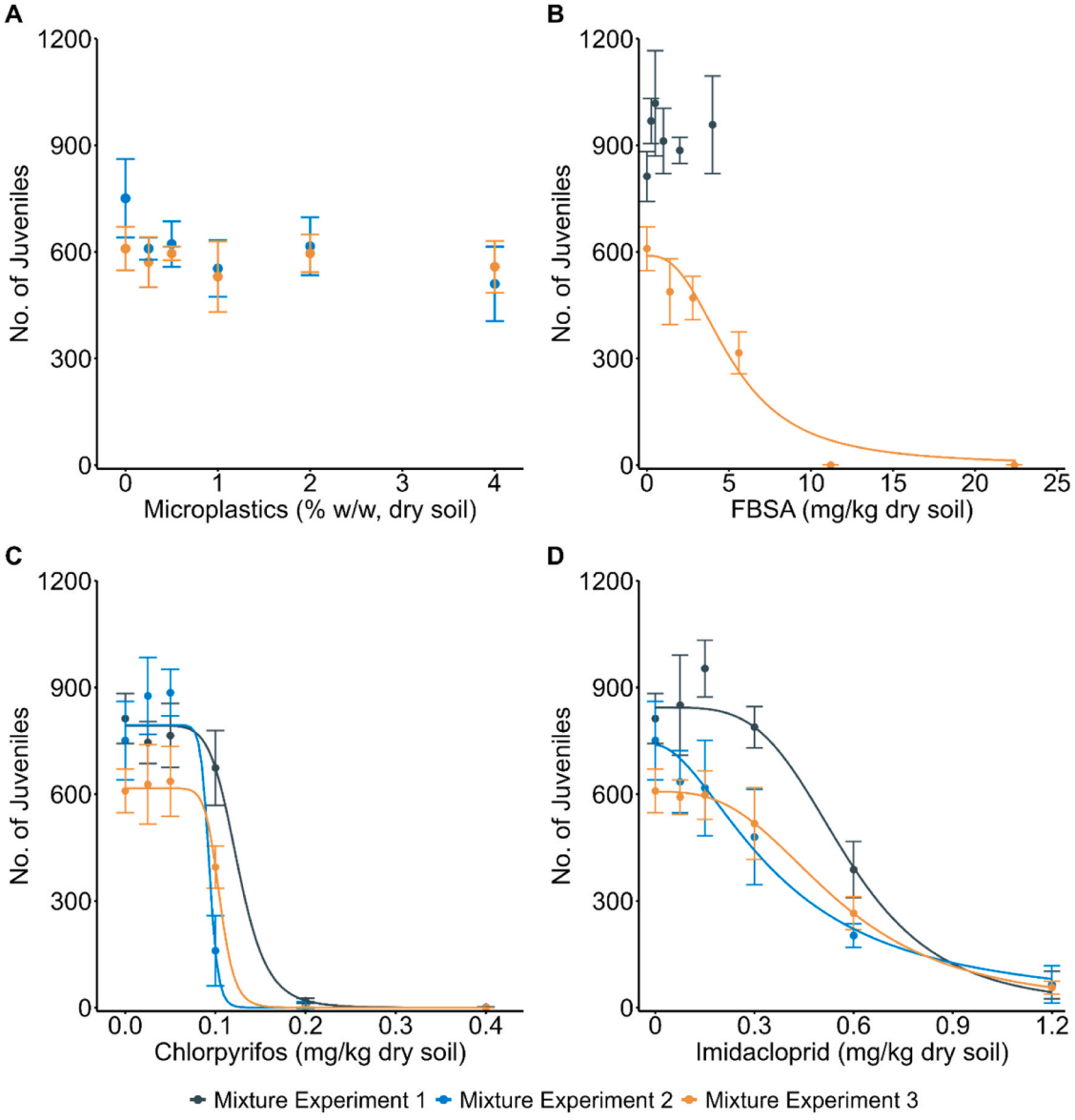

Fig. 1. Dose-response curves for the toxicity of the single compounds, linear low-density polyethylene microplastics (A), perfluorobutane sulfonamide (FBSA; B), chlorpyrifos (C) and imidacloprid (D) in three experiments on the reproduction of *Folsomia candida* in Lufa 2.2 soil. Dots indicate the mean juvenile numbers at the nominal test concentrations, with the error bars indicating standard deviation (n = 5, n = 20 for controls). Lines are fitted using a three-parameter logistic dose-response model. If a line is absent, the data did not allow for the proper modelling of a dose-response relationship.

Table 2
Parameters for the three-parameter logistic dose-response relationships for the effect of the single chemicals on the reproduction of *Folsomia candida* exposed for 3 weeks to linear low-density polyethylene microplastics (MPs), perfluorobutane sulfonamide (FBSA), chlorpyrifos (CPF) or imidacloprid (IMI) in Lufa 2.2 soil. Y_{max} is the maximum asymptote of the dose-response relationship, Slope indicates the steepness of the curve, and EC_{50} the 50 % effect concentration. Additionally, EC_{10}, the 10 % effect concentration, is shown. Asterisks indicate chemicals tested at non-toxic concentrations.

	Y_{max}	Slope	EC_{50}	EC_{10}
Experiment 1				
FBSA*	-	-	-	-
CPF	793	7.76	0.125	0.0942
IMI	844	4.02	0.580	0.336
Experiment 2				
MPs*	-	-	-	-
CPF	794	19.4	0.0931	0.0832
IMI	739	1.80	0.375	0.111
Experiment 3				
MPs*	-	-	-	-
FBSA	589	2.59	5.17	2.22
CPF	616	12.7	0.105	0.088
IMI	607	2.91	0.550	0.259

Table 3
Parameter values describing the binary interactions between the effects of two compounds in mixtures of linear low-density polyethylene microplastics (MPs), perfluorobutane sulfonamide (FBSA), chlorpyrifos (CPF) and imidacloprid (IMI) on the reproduction of *Folsomia candida* in Lufa 2.2 soil. The a-value shows whether the interaction between the two compounds in a mixture is synergistic ($a < 0$) or antagonistic ($a > 0$). The b-value indicates if the best fitting model is dose ratio (DR) or dose level (DL) dependent and the nature of that DR/DL relationship. All parameters are interpreted according to Table 1 in [27]. Asterisks indicate chemicals tested at non-toxic concentrations.

	a-value	b-value (if DR or DL)	Significance of interactions: χ^2 and p-values	Best model fit
Experiment 1				
CPF-FBSA*	1.06	-	$\chi^2_{df=1} = 16.1$, $p < 0.001$	CA with antagonism
CPF-IMI	0.475	-	$\chi^2_{df=1} = 4.20$, $p < 0.05$	CA with antagonism
FBSA*-IMI	-1.83	0.613 – DL	$\chi^2_{df=1} = 4.86$, $p < 0.05$	CA with dose level dependent magnitude of synergism
Experiment 2				
CPF-MPs*	0.49	-	$\chi^2_{df=1} = 7.72$, $p < 0.01$	CA with antagonism
CPF-IMI	1.55	-	$\chi^2_{df=1} = 74.9$, $p < 0.001$	CA with antagonism
MPs*-IMI	5.07	0.511 - DL	$\chi^2_{df=1} = 7.19$, $p < 0.01$	CA with dose level dependent magnitude of antagonism
Experiment 3				
MPs*-FBSA	3.93	-	$\chi^2_{df=1} = 58.7$, $p < 0.001$	CA with antagonism
MPs*-CPF	1.58	-	$\chi^2_{df=1} = 62.9$, $p < 0.001$	CA with antagonism
FBSA-CPF	1.06	-	$\chi^2_{df=1} = 53.7$, $p < 0.001$	CA with antagonism
MPs*-IMI	1.25	-	$\chi^2_{df=1} = 8.32$, $p < 0.01$	CA with antagonism
FBSA-IMI	1.10	-	$\chi^2_{df=1} = 36.1$, $p < 0.001$	CA with antagonism

imidacloprid:FBSA compared to imidacloprid alone, even though FBSA alone was not toxic at any of the tested concentrations. In contrast, all the other binary interactions between the MPs, FBSA, chlorpyrifos and imidacloprid were antagonistic, with the best fitting model being concentration addition with antagonism. Only the magnitude of antagonism between the MPs and imidacloprid in Experiment 2 was dose-level dependent. At its toxic concentrations, FBSA also showed antagonistic interactions with both insecticides.

3.6. Toxicity of ternary mixtures

Significant interactions were observed in all ternary mixtures ($\chi^2_{df=1} = 23.7 - 125$, $p < 0.001$). The overall interactions were the same in all ternary mixtures tested, with the best model fit resulting from CA with antagonism and additional ternary antagonism, meaning there was additional antagonism (*a4* overall, Table 4; CA + S/A + S/A, Table 5) compared to the antagonism expected based on the binary interactions (a-values, Table 3; CA + SA, Table 5). To gain insight into ratio-specific ternary interactions, the additional ternary S/A models were not only fitted to all data, but also to each individual tested ternary chemical ratio (i.e. fitting individual *a4s* for each of the four different ratios). The values of the individual *a4s* (Table 4) showed that the only non-antagonistic ternary interaction was for chlorpyrifos combined with FBSA at non-toxic concentrations and imidacloprid, in the concentration ratio of 20:20:60, where the a4 being negative shows the additional ternary interaction was synergistic. Despite this ratio-specific synergistic interaction, the remaining ratios were so strongly antagonistic that the overall additional ternary interaction was antagonistic. Comparing springtail reproduction modelled using the CA, CA + S/A and CA + S/A + S/A interactions with the measured reproduction data provides insight into the magnitude of the synergism/antagonism in all ternary mixtures, in terms of biological effects (Fig. 4). To this end, in Table 5, the level of deviation of the model compared to the measured value is indicated by color. The modelled value is colored blue if it is lower than the measured value. The modelled value is colored red if it is higher than the measured value. Overall, the CA modelled value was mostly below the measured value, but the CA + S/A + S/A models gave a slightly better approximation of the measured values, as indicated by the lighter colors in Table 5.

Fig. 5 shows what happens to the toxicity of an equitoxic mixture of the other two compounds when the fraction of one component in the ternary mixture increases, as described by Cedergreen et al. [9]. It can be interpreted as an isobole of the isoplane, at 50 % effect concentration of the ternary mixture. The solid lines represent the overall model prediction based on the binary interactions, and the dashed lines show the extra ternary interactions identified by the fitting of the overall *a4*. The symbols represent the EC_{50} resulting from the individual ternary mixture ratios (CA + S/A + individual S/A). For example, in Fig. 5A, the solid orange line, from the left to the right, shows how an increasing proportion of chlorpyrifos in a ternary mixture where FBSA and imidacloprid are equitoxic, affect the toxicity predicted based on the binary interactions. In this case, it shows that the ternary interaction (predicted from binary interactions) switches from synergism to antagonism at around 40 % chlorpyrifos in the mixture. However, the orange point indicates that when 60 % chlorpyrifos is present in the mixture, the ratio-specific antagonism was so strong that approximately 2.5 TU of the mixture would be needed to cause 50 % effect. In this case, the orange line is much lower than the point, showing that the overall model is averaging out the ratio-specific synergism observed when little chlorpyrifos is present in the mixture with the antagonism in the remaining ratios.

Fig. 5B shows that very strong ratio-specific antagonism occurs when 60 % of imidacloprid is present in a ternary mixture where the MPs and chlorpyrifos are equitoxic. The strongest ratio-specific antagonism in Experiment 3 was observed when 60 % of FBSA (at toxic concentrations)

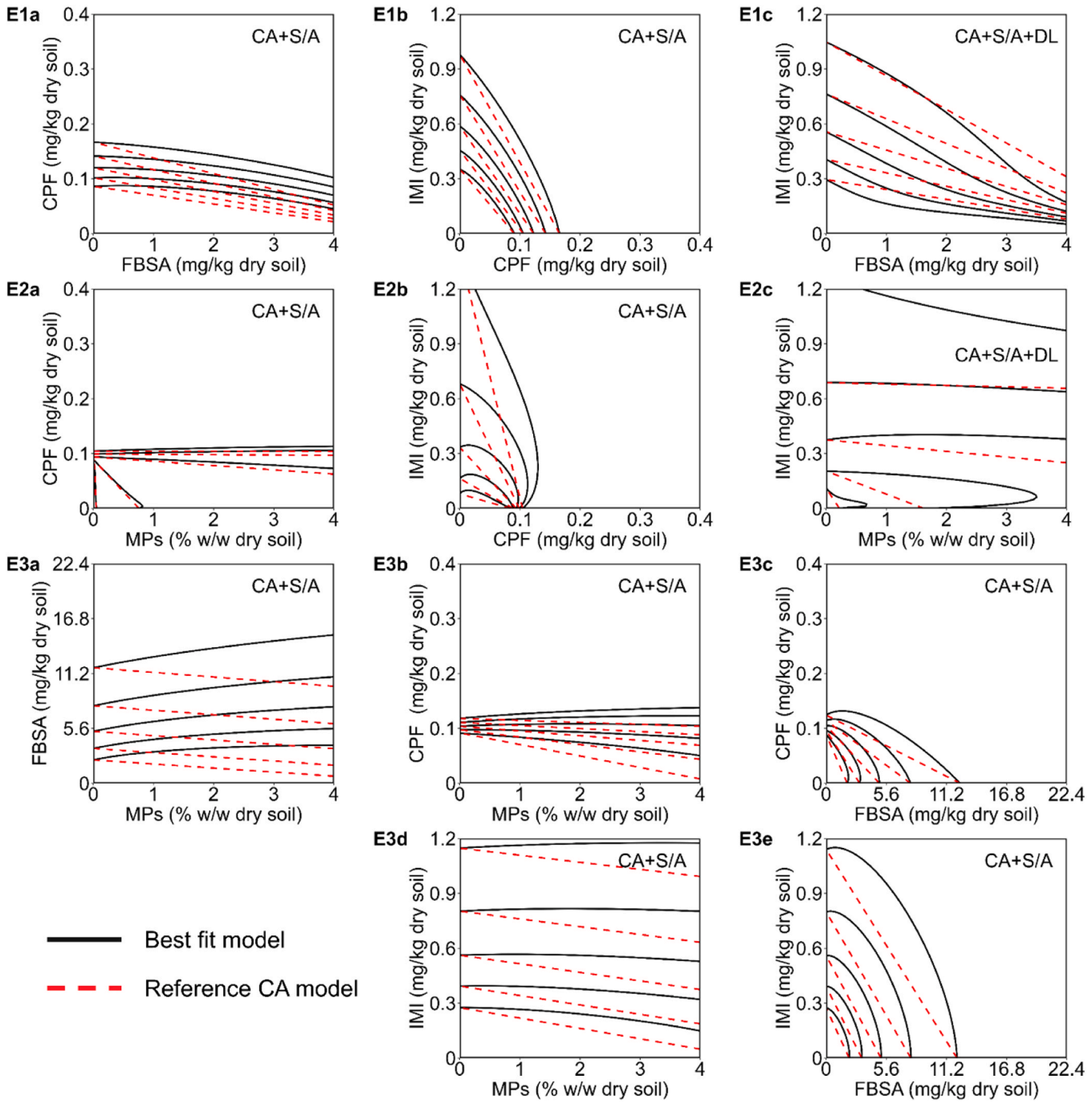

Fig. 2. Isoboles at 10 %, 25 %, 50 %, 75 %, and 90 % effect levels for the binary mixtures of linear low density polyethylene microplastics (MPs), perfluorobutane sulfonamide (FBSA), chlorpyrifos (CPF) and imidacloprid (IMI) on the reproduction of *Folsomia candida* in Lufa 2.2 soil in the three experiments (E1a-c, E2a-c, and E3a-e). The best fitting model to describe the interactions between the two compounds is provided in the top right corner of each graph. CA = Concentration Addition, S/A = Synergism/Antagonism, and DL = Dose Level dependency.

is present in ternary mixtures where the MPs are equitoxic with either chlorpyrifos or imidacloprid (Fig. 5 C and 5D).

4. Discussion

The aim of this study was to assess how short-chain PFAS and MPs as ubiquitous pollutants affect potencies of highly toxic chemicals in mixtures. Interactions were observed in all mixtures involving FBSA and MPs at non-toxic concentrations. FBSA at non-toxic concentrations showed synergistic interactions with imidacloprid, but antagonistic with chlorpyrifos. This synergism was also observed in the ternary mixtures dominated by FBSA and imidacloprid, but not in those dominated by chlorpyrifos. FBSA at toxic concentrations showed antagonistic interactions with all the other compounds. MPs showed antagonistic interactions in all binary and ternary mixtures. As these antagonistic interactions were quite strong for all compounds, the influence of lipophilicity on the interaction with MPs could not be determined.

4.1. Toxicity of single compounds

Dose response curves for the single compounds resulted in EC_{50} values within the ranges expected from the literature [12,46]. The lack

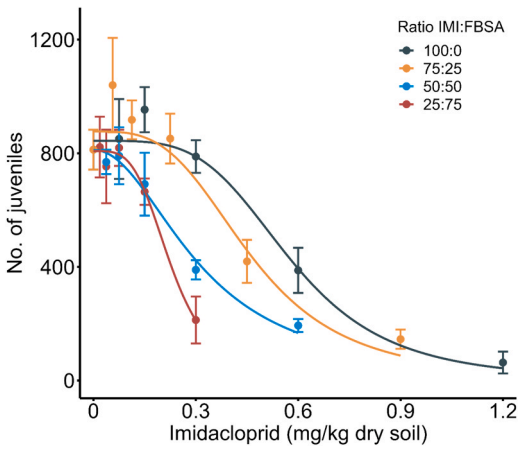

Fig. 3. Dose-response relationships for the toxicity of imidacloprid to the reproduction of *Folsomia candida* after 21 days of exposure to the insecticide alone or in mixtures at different ratios with concentrations of perfluorobutane sulfonamide (FBSA) at non-toxic levels in Lufa 2.2 soil.

Table 4
Parameters of the ternary interactions between the effect of three compounds in mixtures of linear low-density polyethylene microplastics (MPs), perfluorobutane sulfonamide (FBSA), chlorpyrifos (CPF) and imidacloprid (IMI) tested for their effects on the reproduction of *Folsomia candida* in Lufa 2.2 soil. The *a*-value shows whether the interaction between the two compounds in that mixture is synergistic ($a < 0$) or antagonistic ($a > 0$); *a4* gives the overall indication of the synergism/antagonism of the additional ternary interaction when the model was fitted to all single, binary and ternary data from a mixture toxicity experiment. The *a4*-values of the different ratios indicate the synergism/antagonism when the model was fitted to all single, binary and a single ratio of the ternary data, providing insight into the ratio-specific interactions of the three compounds in the mixture. Asterisks in the table header indicate chemicals tested at non-toxic concentrations.

Ternary interaction	CPF:FBSA*: IMI	CPF:MPs*: IMI	MPs*:FBSA: CPF	MPs*:FBSA: IMI
a4 overall	10.8	54.6	32.7	21.0
a4 33:33:33	2.43	40.7	24.2	35.6
a4 60:20:20	35.7	64.3	31.7	14.9
a4 20:60:20	12.2	41.1	151	74.6
a4 20:20:60	-3.39	144	47.3	26.3

of effects of MPs on the reproduction of *Folsomia candida* in Lufa 2.2 soil confirms our earlier findings [56,57,58]. FBSA was tested both at non-toxic concentrations (Experiment 1) and toxic concentrations (Experiment 3). Although no toxicity data of FBSA is currently available for adult *F. candida*, the EC_{50} value obtained from Experiment 3 (5.17 mg/kg dry soil) was consistent with the value from the range-finding test (5.58 mg/kg dry soil). EC_{50} values for chlorpyrifos derived from all three experiments (0.125, 0.0931 and 0.105 mg/kg dry soil) only differed by a factor of 1.34 and fall in the range (0.045–0.13 mg/kg dry soil) reported in literature, although those tests were started with juveniles [23,45,55]. The EC_{50} values for imidacloprid (0.58, 0.375 and 0.55 mg/kg dry soil) varied by a factor of 1.55 across the three experiments, but are in good agreement with previous studies (0.264 – 0.82 mg/kg dry soil) [17,32,54]. The currently obtained EC50 values for imidacloprid for adult springtails were slightly higher than the values from the literature for juveniles, which can thus be explained by differences in sensitivity between the different life stages [17].

4.2. Toxicity of binary mixtures

MPs acted antagonistically in all binary mixtures with the other compounds, contradicting the assumption based on aquatic literature that they may act as contaminant transporters thereby increasing their toxicity. This is contrasting to our observations of MPs being associated with a lowering of chemical toxicity. It could be assumed that this lowering of toxicity is caused by adsorption of chemicals to the plastic particles. Such binding may be due to the virgin nature of the MPs used in the study, and may not occur in a scenario where aged MPs are loaded with chemicals, e.g. MPs in sewage sludge. Dedicated research would be needed to study how the interactions between chemicals and unloaded MPs affect toxicity, compared to exposure to MPs pre-loaded with the same chemicals. A study by, Palacio-Cortes et al. [41], found after exposure of chironomid larva to polybrominated diphenyl ethers (PBDE) both with and without MPs present, that microplastics significantly reduced PBDE concentrations in chironomid tissues for all congeners at all concentrations [41]. However, this study was performed in water using different chemicals and not including sediment. As a consequence, also because of the difference in feeding behavior between soil and aquatic organisms, the two studies could not reliably be compared. It also remains unclear why this antagonistic interaction with MPs was seen for both chlorpyrifos and imidacloprid, as it would suggest a similar degree of binding to the plastic particles. A strong binding was expected for the lipophilic chlorpyrifos (log Kow = 4.96), but not for the hydrophilic imidacloprid (log K_{ow} = 0.57). It should be noted, however, that the same holds for the binding of both compounds to the organic matter in the Lufa 2.2 test soil. Since imidacloprid is known to also bind to clay particles [38], surface charge of the MPs may have contributed to its stronger than expected binding. A multicompartment partitioning model might perhaps shed more light on this, but such a model cannot be applied without knowing the partitioning coefficients for chlorpyrifos and imidacloprid on the LLDPE MPs used in this study.

Antagonism was also shown in all mixtures with FBSA, except for the mixture of FBSA at non-toxic concentrations and imidacloprid, where synergism was observed. Since the mode of action of FBSA has rarely been studied, we could only speculate on what caused the observed toxicity interactions. The C8 homologue, perfluorooctane sulfonamide (FOSA), has been reported to affect the immune system and interact with CYP-450 enzymes [40,10]. If it is assumed that FBSA induces similar effects, the synergistic interactions between FBSA at non-toxic concentration and imidacloprid may be explained by the hypothesized effects of FBSA on the immune system that could decrease the detoxification of imidacloprid. The unexpected antagonism between FBSA at toxic concentrations and imidacloprid is likely driven by a different, yet unknown mechanism. The hypothesized interaction with CYP-450 enzymes could cause FBSA to act antagonistically with chlorpyrifos by lowering its bioactivation through CYP-450. The interaction between chlorpyrifos and imidacloprid was consistently antagonistic. Chlorpyrifos could inhibit nAChR binding of imidacloprid *in vivo*, decreasing its toxicity, while imidacloprid could inhibit CYP-450 enzymes, lowering the bioactivation of chlorpyrifos [47,68]. These reciprocal effects could contribute to the observed antagonism between the two insecticides. Nonetheless, the hypothesized mechanisms of interaction require further investigation.

4.3. Toxicity of ternary mixtures

The ternary mixtures showed similar interactions as observed in the binary mixtures: the best fitting model for all mixtures was CA + S/A + S/A with additional ternary antagonism. In general, including knowledge from the binary interaction models provided improved predictions of joint ternary effects (CA + S/A) compared to the pure CA model, which were much closer to the observed overall ternary interactions seen in all three experiments of this study. The overall additional ternary antagonism fits well with the interactions predicted from the binary mixtures, except for the mixture of FBSA at non-toxic concentrations with chlorpyrifos and imidacloprid. In this mixture, the synergism was missed in the overall analysis where ternary antagonism was observed.

Table 5
Modelled reproduction (number of juveniles produced) of *Folsomia candida* exposed to ternary mixtures of linear low density polyethylene microplastics (MPs), perfluorobutane sulfonamide (FBSA), chlorpyrifos (CPF) and imidacloprid (IMI) in Lufa 2.2 soil. Toxic units of specific ratios are given with the measured reproduction (Raw data). Modelled reproduction is presented for the concentration addition model (CA), concentration addition model with synergism/antagonism (CA + S/A) expected based on the binary interactions, concentration addition model with synergism/antagonism with overall additional ternary synergism/antagonism (overall CA + S/A + S/A) and the concentration addition model with synergism/antagonism with additional ternary synergism/antagonism when modelled over the single, binary and a specific ratio of ternary data (individual CA + S/A + S/A). Colors are graded by the percentage level of deviation of the model from the measured values for a specific exposure concentration, with blue indicating that the modelled value is lower than the measured value and red indicating that the modelled value is higher than the measured value and underestimates the level of effect that was observed in the measured data. Asterisks in the table header indicate chemicals tested at non-toxic concentrations.

CPF:FBSA*:IMI					Overall	Individual
	Toxic units	Raw data (x̄ ± sd)	CA	CA+S/A	CA+S/A+S/A	CA+S/A+S/A
33:33:33	1 TU	597 ± 117	848	848	864	853
	2 TU	492 ± 62.4	415	416	671	480
	4 TU	107 ± 40.9	11.0	11.0	48.6	15.6
60:20:20	1 TU	777 ± 49.4	791	791	833	857
	2 TU	453 ± 54.5	244	244	227	448
	4 TU	2.40 ± 2.06	11.2	11.2	3.33	11.0
20:60:20	1 TU	777 ± 119	867	866	871	871
	2 TU	727 ± 70.9	732	724	855	858
	4 TU	444 ± 78.2	73.3	67.4	371	438
20:20:60	1 TU	590 ± 77.6	822	821	847	818
	2 TU	385 ± 64.0	426	420	540	349
	4 TU	195 ± 60.4	39.3	38.2	47.6	18.2
CPF:MPs*:IMI						
33:33:33	1 TU	622 ± 81.4	531	535	619	603
	2 TU	490 ± 87.2	221	232	483	435
	4 TU	3.40 ± 3.38	0.47	0.65	121	60.2
60:20:20	1 TU	588 ± 53.7	511	512	576	584
	2 TU	27.4 ± 29.9	3.41	3.66	55.9	78.9
	4 TU	2.80 ± 2.32	0	0	0	0
20:60:20	1 TU	642 ± 99.4	562	569	663	646
	2 TU	683 ± 25.5	432	448	618	592
	4 TU	605 ± 50.5	107	137	546	496
20:20:60	1 TU	608 ± 59.0	483	485	547	617
	2 TU	561 ± 48.7	223	225	316	456
	4 TU	100 ± 28.3	30.8	31.8	75.4	190
MPs*:FBSA:CPF						
33:33:33	1 TU	487 ± 82.0	470	474	543	530
	2 TU	236 ± 46.8	96.9	102	269	221
	4 TU	24.6 ± 5.43	0.423	0.497	9.27	4.90
60:20:20	1 TU	543 ± 111	555	559	588	588
	2 TU	516 ± 72.8	341	365	560	558
	4 TU	366 ± 56.1	27.9	35.6	368	357
20:60:20	1 TU	536 ± 58.8	361	363	406	516
	2 TU	274 ± 41.9	79.5	80.3	111	270
	4 TU	0.400 ± 0.800	4.24	4.32	7.53	39.7
20:20:60	1 TU	548 ± 40.1	460	462	517	534
	2 TU	35.8 ± 13.6	4.90	5.17	24.7	44.0
	4 TU	2.60 ± 3.32	0	0	0	0
MPs:FBSA:IMI						
33:33:33	1 TU	510 ± 36.1	492	495	531	548
	2 TU	458 ± 30.1	250	256	341	397
	4 TU	70.2 ± 17.2	55.5	57.6	96.3	134
60:20:20	1 TU	462 ± 50.1	552	556	577	573
	2 TU	488 ± 32.6	411	427	532	511
	4 TU	308 ± 43.5	146	161	345	289
20:60:20	1 TU	460 ± 71.1	396	397	415	456
	2 TU	219 ± 57.2	140	140	156	202
	4 TU	0.400 ± 0.800	26	26	30	42.7
20:20:60	1 TU	548 ± 84.9	501	503	528	534
	2 TU	363 ± 55.5	254	258	320	336
	4 TU	53.2 ± 43.3	52.3	53.5	78.5	86.2

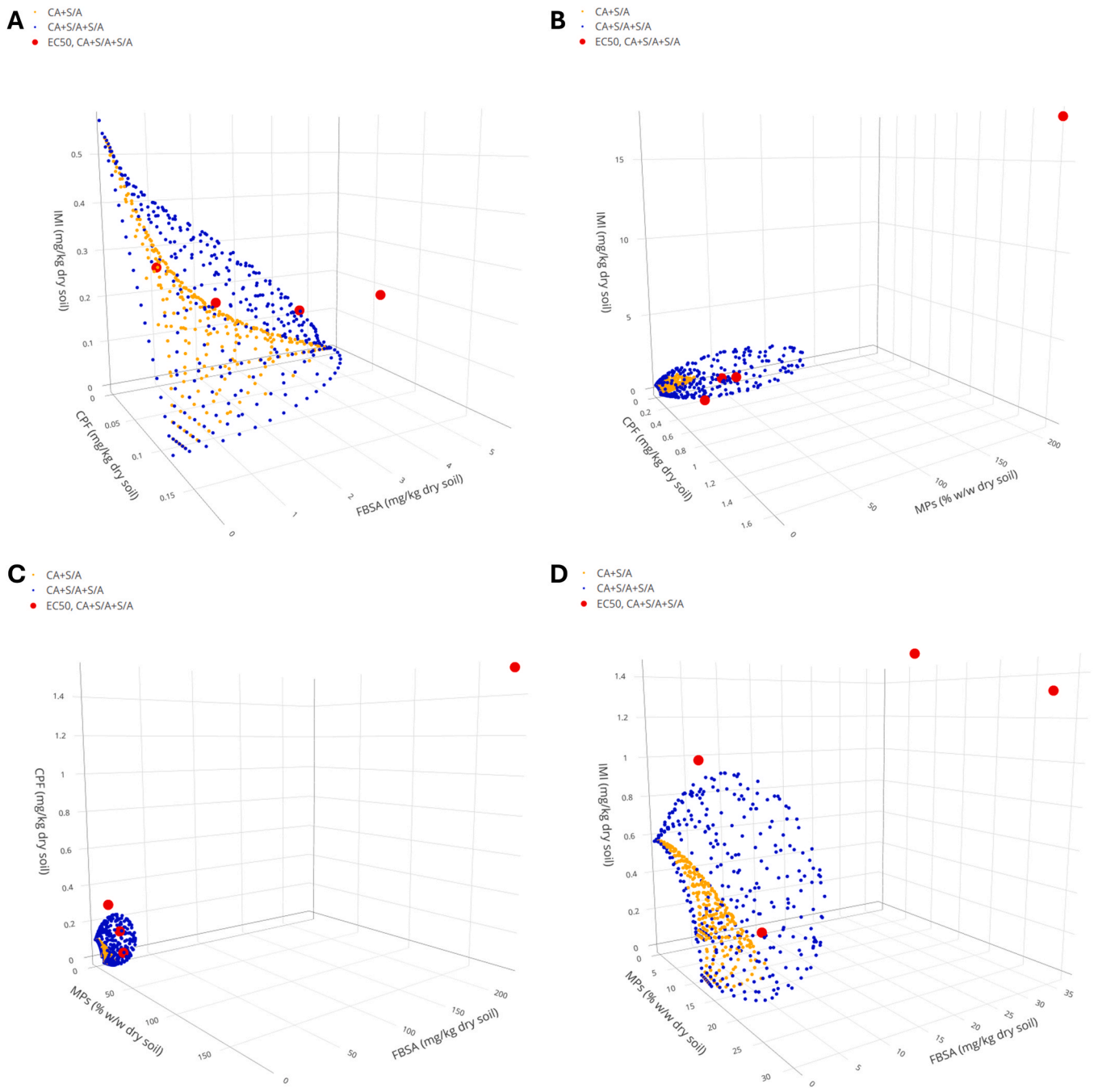

Fig. 4. Ternary isoplane (3D isobole) at 50 % effect level for the effects of ternary mixtures of linear low density polyethylene microplastics (MPs), perfluorobutane sulfonamide (FBSA), chlorpyrifos (CPF) and imidacloprid (IMI) on the reproduction of *Folsomia candida* in Lufa 2.2 soil in the three experiments: A) FBSA at non-toxic concentrations combined with chlorpyrifos and imidacloprid, B) MPs at non-toxic concentrations combined with chlorpyrifos and imidacloprid, C) MPs at non-toxic concentrations combined with FBSA at toxic concentrations and chlorpyrifos, D) MPs at non-toxic concentrations combined with FBSA at toxic concentrations and imidacloprid. The yellow grid represents the concentration addition + synergism/antagonism (CA + S/A) model, indicating what the ternary interactions are predicted to be, based on the data of the single compounds and binary mixtures. The blue grid represents the CA + S/A + S/A model, indicating the true ternary interaction, fit to all single compound, binary mixture and ternary mixture data. The red dots indicate the 50 % effect concentration, when the ternary interactions are fitted to all single compound and binary data, as well as a single ternary ratio, instead of all ternary data, aiding in recognizing ratio-specific interactions in ternary mixtures.

The CA + S/A + S/A model indicates the real magnitude of S/A in the ternary mixture compared to what can be predicted from the binary interactions alone. However, it provides insight only into the overall interaction, as fitted over all data; i.e., over all compounds in the mixtures, at all dose levels and dose ratios of the compounds. As this represents an overall interaction, this may "average out" interactions at specific ratios or specific influence of dominant compounds. To explore this, also the "CA + S/A + individual S/A" values were calculated using the single, binary and each of the individual ternary ratio data (Tables 3 and 4). This allows comparison of the model fit using all ternary datapoints and model fits when only data from a single ternary ratio is included, providing insight into how interactions at each specific ratio of three compounds may differ from the overall interaction which is effectively an average of the individual ratios. Doing so, allows

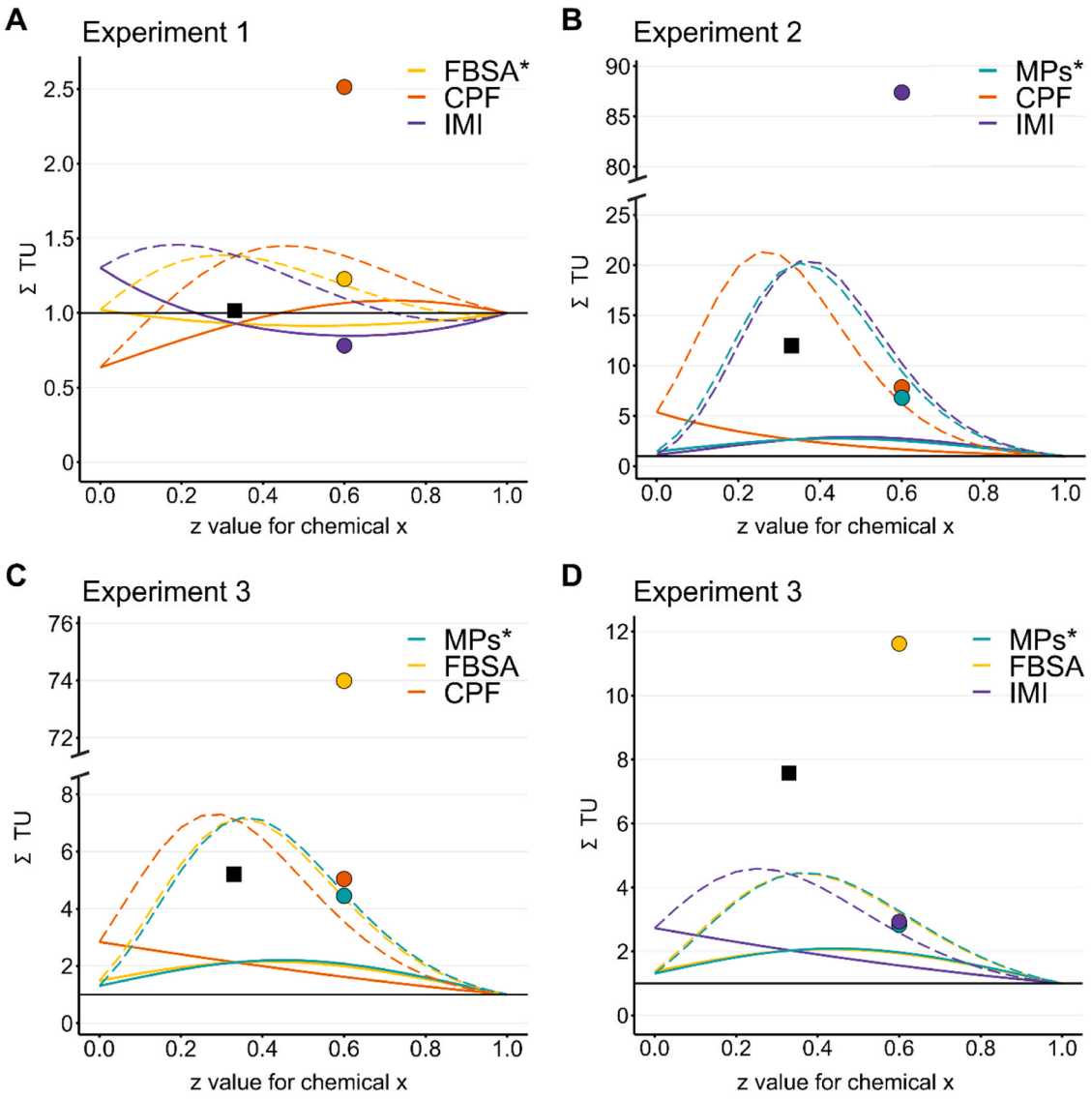

Fig. 5. The transects of the EC_{50} isoplanes for the four ternary mixtures tested in this study for their effects on the reproduction of the adult springtail *Folsomia candida* in Lufa 2.2 soil, shown as the sum of toxic units (TU) for the mixture that caused 50 % of effects as a function of the fraction (z) for each of the three components, where the remaining components are equitoxic. Solid lines show the transects for the ternary model including only the binary interactions (using interaction terms *a1-a3*, concentration addition (CA)+synergism/antagonism (S/A)). The dashed lines represent the transects for the ternary model including the binary interactions as well as an additional ternary interaction parameter (*a4*) describing the ternary interactions (CA + S/A + S/A); see text for further explanation. Symbols show the EC_{50} values of the ternary mixture at each experimental mixture ratio (individual *a4*), calculated by the CA + S/A + S/A model fitted over the single, binary and a single ratio of the ternary data. The mixtures containing the 60 % standard exposure level (SEL) of linear low density polyethylene microplastics (MPs), perfluorobutane sulfonamide (FBSA), chlorpyrifos (CPF), and imidacloprid (IMI) according to the individual *a4* model, are given as dots in corresponding colors, while the 33:33:33 % mixture is given as black squares. The reference CA model is given as a black solid line at $\sum TU = 1$. Asterisks in the figure legends indicate chemicals tested at non-toxic concentrations.

identification of dose-ratio specific interactions that may otherwise be overlooked when only looking at the overall ternary interaction. In the case of chlorpyrifos with FBSA at non-toxic concentrations and imidacloprid at a 20:20:60 ratio, additional ternary synergism could be observed, despite the overall additional ternary interaction being antagonistic.

4.4. Implications for risk assessment of mixtures

It is worrying that non-toxic concentrations of ubiquitously occurring chemicals modified the toxicity of other compounds. The exact ecological implications are, however, difficult to predict from this laboratory study. It is also worrying that the level of ternary interactions could not be reliably predicted from binary interactions. The level of overall antagonism observed for the ternary mixtures is higher than expected based solely on the binary interactions. The CA model is generally precautionary and therefore presents a solid basis to safely assess the risk of the currently selected mixtures. This is, however, not necessarily the case for other, untested mixtures.

Moreover, strong ternary antagonisms may mask CA or even synergisms occurring in binary mixtures. In the data presented in this study, this is highlighted by the synergistic interaction in the 20:20:60 chlorpyrifos:FBSA (at non-toxic concentrations):IMI mixture, being overshadowed in the overall ternary interaction analysis by the strong antagonistic interaction between FBSA and chlorpyrifos at other ratios of the mixture. In this case, the CA model is not precautionary for all ratios of the mixture.

This indicates that to reliably assess the effect of complex mixtures,

not only the synergism/antagonism of the mixture as a whole should be considered, but also the interactions at specific ratios. The large interactions observed, some of which were synergistic, also demonstrate the relevance of including concentrations of ubiquitous pollutants at non-toxic concentrations in the risk assessment of mixtures.

The present study emphasizes the relevance of including MPs and FBSA in the risk assessment of mixtures. However, despite being ubiquitous and therefore always present in environmental mixtures, in the current proposal to include a mixture assessment factor (MAF) in REACH legislation [36], these compounds are left out of mixture risk assessment. Although primary MPs are included in REACH, most MPs are secondary MPs: fragments of larger plastic products. These plastic products are considered oil products and therefore exempted from REACH. Many PFAS are considered transformation products and non-isolated intermediates, therefore also mostly left out of REACH legislation thus far [15]. The MAF as proposed therefore does not include all relevant components of environmental mixtures, and this study shows the relevance to include ubiquitous substances like secondary MPs and PFAS, even when environmentally relevant concentrations are at non-toxic levels.

Another reason why these ubiquitous substances may not work in the MAF would be the assumptions for mixtures that are covered by the currently proposed MAF: 1) the chemicals in the mixture behave according to the concentration addition (CA) model, 2) the concentration-additive risk of a mixture is adequately estimated as the sum of individual Risk Quotients (RQ), 3) the RQs of the mixture components are independent, and 4) the mixture of interest is well defined [2].

This study shows that the assumption that the chemicals in the mixture behave according to the CA model may not always be valid. Backhaus [2] justified this assumption in view of the empirical evidence at hand, showing significant deviations from CA are rare in relevant multi-component mixtures, something that is challenged by the large interactions caused by the two ubiquitous compounds tested in this study. Another challenge with the assumptions of the proposed MAF is that due to technical limitations, the concentrations of MPs and most PFAS in soil are often unknown, hampering a proper definition of the mixture with current lab techniques. Therefore, the currently proposed MAF at this moment seems unsuitable for dealing with these ubiquitous constituents of environmental mixtures.

5. Conclusions

In the present study, MPs and FBSA both affected the toxicity of chlorpyrifos and imidacloprid, in all binary and ternary mixtures, even at non-toxic concentrations of the MPs and FBSA. For MPs, this was likely due to the binding of chemicals to the particles, which may effectively have lowered their bioavailability. For FBSA, the impact on the toxicity of the insecticides may be through effects on the immune system and CYP-450 enzymes of the springtails, although the mechanisms behind these interactions were not further investigated. FBSA, at non-toxic concentrations, showed a dose-dependent synergism with imidacloprid, while being antagonistic with chlorpyrifos in the binary mixtures. In the ternary mixture of FBSA at non-toxic concentrations, chlorpyrifos, and imidacloprid, ratio-specific synergism was observed, while the overall ternary interaction was antagonistic. The MPs, at non-toxic concentrations, were antagonistic with chlorpyrifos and imidacloprid in binary mixtures, as well as with both insecticides in the ternary mixture. FBSA at toxic concentrations was antagonistic with both insecticides in binary mixtures. The interactions of MPs and FBSA at its toxic concentrations with either insecticide were also antagonistic. The antagonistic interactions with MPs were very strong for all compounds, with no differences depending on their lipophilicity being observed. Given the strong interactions observed in this study, it is of high relevance to include ubiquitous substances like MPs and PFAS in the risk assessment of chemical mixtures. The results also showed that ternary interactions may not reliably be predicted from binary interactions, as ratio-specific interactions may be overlooked and the magnitude of ternary interactions may be underestimated. Therefore, experimental data from well-designed mixture toxicity studies is needed for a realistic risk assessment of complex chemical mixtures.

Environmental implications

The study presented in this manuscript provides insight into the effects of two ubiquitous contaminants, microplastics and a PFAS, on the toxicity of two insecticides in mixtures. Microplastic and PFAS at non-toxic concentrations, as well as toxic concentrations of the PFAS were included in mixtures and proved mostly antagonistic with the other compounds. However, synergistic interactions also occurred. These interactions could not be reliably predicted from the interactions in binary mixtures, therefore, ternary mixture experiments are needed to provide reliable insights on ternary interactions. Finally, we discuss the implications of this work for the risk assessment of mixtures.

CRediT authorship contribution statement

Ge Xie: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Claus Svendsen:** Writing – review & editing, Supervision, Funding acquisition, Formal analysis. **Kraak Michiel H.S.:** Writing – review & editing, Supervision. **Lotte de Jeu:** Investigation. **Nienke C. Schut:** Investigation. **Eva Sprokkereef:** Investigation. **Rachel Hurley:** Resources, Funding acquisition. **Annemarie P. van Wezel:** Writing – review & editing. **Cornelis A.M. van Gestel:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Sam van Loon:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2025.140493.

Data availability

The raw survival and reproduction data from this study are available on the open-access research data repository Zenodo. The datasets can be accessed at: https://doi.org/10.5281/zenodo.14961673.

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