Mixture toxicity of herbicides with dissimilar modes of action to *Myriophyllum spicatum*

ABSTRACT

Considering the vital role of rooted macrophytes in the aquatic ecosystem, validating assumptions on the interactive effects of herbicides with different modes of action at an environmentally relevant mixture ratio is necessary. Here, we investigated the effects of diflufenican (a carotenoid biosynthesis inhibitor) and iodosulfuron-methyl-sodium (an acetolactate synthase inhibitor) in a 14-day growth inhibition experiment with *Myriophyllum spicatum*, wherein single compounds and their combination were tested in parallel (n=84). The assessment was done using three different methods: significance testing, Model Deviation Ratio (MDR), and Mixture Interaction Factor (MIF). Interactions relative to both concentration addition (CA) and independent action (IA) were assessed via significance testing. And this revealed that diflufenican and iodosulfuron-methyl-sodium acted antagonistically relative to both models for fresh weight and total shoot length ($p<0.05$) and there was slight synergism for the number of side shoots ($p<0.001$) relative to CA. The MDR and MIF can only assess interactions relative to the CA model. According to MDR the mixture appeared to show no interaction (neither antagonistic nor synergistic) while the MIF method revealed that the compounds acted antagonistically for fresh weight and there was a slight synergistic trend for total shoot length and number of side shoots. We conclude that inferences about mixture toxicity interactions are method and endpoint dependent, which can have implications for regulatory mixtures assessment.

Keywords: herbicide; mixture; diflufenican; iodosulfuron-methyl-sodium; Myriophyllum; risk assessment

*Address correspondence to ma.fusilero@ugent.be

INTRODUCTION

Co-occurrence of active substances of different pesticides in the surface water is expected as farmers follow crop-specific treatment programs. Pesticides are applied sequentially or in parallel depending on the crop growth stage and pest (weeds, insects, and diseases) pressure and farmers also use tank mixtures of different pesticides. This leads to multiple pesticides entering the freshwater systems through drift, run-off, or drainage from agricultural fields.

The importance of pesticide mixture toxicity assessment has been studied and reviewed extensively (Abendroth et al., 2011; Arts et al., 2006; Belgers et al., 2011; Cedergreen, 2014; de Zwart & Posthuma, 2005; Diepens et al., 2017; Faust et al., 2001; Feller et al., 2012; Gustavsson et al., 2017; Knauert et al., 2010, 2008; Knežević et al., 2016; Schreiner et al., 2016; Sørensen et al., 2007; Suresh Kumar & Han, 2011; van Wijngaarden & Arts, 2018; Wendt-Rasch et al., 2004). At first instance, two reference models are considered: Concentration Addition (CA) model, which often holds true for compounds with similar modes of action, and Independent Action (IA) model or response addition, which is considered as a valid model for compounds with dissimilar modes of action. A second level assessment extends the function of these models to determine the interaction of chemicals with respect to CA or IA. The interaction, if present, could either be synergistic or antagonistic.

It was noted by Cedergreen and Rasmussen (Cedergreen & Rasmussen, 2017), that approximately 90% of pesticide mixtures could be described by CA or IA which still leaves

10% deviating from the model. There are likely synergistic or antagonistic interactions that induce effects higher or lower than predicted by CA and IA mixture models. The CA and IA models have been extensively studied for aquatic invertebrates (Deruytter et al., 2017; Hochmuth et al., 2014; Nys et al., 2015), primary producers algae , and floating plant *Lemna* . We, however, found that there is a gap for evaluating and predicting the mixture toxicity of pesticides for rooted aquatic plants, such as *Myriophyllum spicatum*.

Macrophytes influence the physical, chemical, and biological factors in an aquatic ecosystem. Their roles include an increase in habitat complexity thereby increasing surface availability to colonizing microrganisms and increasing species richness ((Thomaz & Cunha, 2010). *M. spicatum* is the standard test species considered as a model organism for submerged dicotyledonous macrophyte due to its practicality in the laboratory and sensitivity to various herbicides (EFSA Panel on Plant Protection Products and their Residues (PPR), 2013a; Organisation for Economic Co-operation and Development, 2014).

Our focal herbicides in the present study are among the most commonly used in cereal production in the European Union. Usage was extrapolated from marketing data on sales (Kynetec, 2017)to select commonly used herbicides, having different modes of action, which are narrowed down to diflufenican (carotenoid synthesis inhibitor) and iodosulfuron-methyl-sodium (inhibits acetolactate synthase).

Standard pesticide mixture risk assessment (EFSA Panel on Plant Protection Products and their Residues (PPR), 2013b) uses endpoints from studies performed under Good Laboratory Practice (GLP) which could be subject to temporal and spatial variability masking small interactions. This highlights the importance of parallel testing for mixture toxicity to validate the current risk assessment scheme.

The objective of this research is to examine the effects of the binary herbicide mixture diflufenican and iodosulfuron-methyl-sodium on *M. spicatum* .The two compounds have

different modes of action and we hypothesize that there could be interaction relative to IA. To test this, we calculated their Predicted Environmental Concentration in surface water, PECsw, using the FOCUS model (FOCUS, 2012) and used this as a basis for selecting the mixture ratio. To mimic environmental exposure, a water-sediment test system was used (Organisation for Economic Co-operation and Development, 2004) . Experiments were designed to allow valid statistical descriptions of the entire concentration-response relationship. Observed effects and effect concentrations of mixtures were compared to predictions of mixture toxicity. The predictions were calculated from the concentration-response functions of individual compounds applying the concepts of CA and IA.

MATERIALS AND METHODS

M. spicatum culture and maintenance

Fresh axenic shoots (ca. 6 cm in length) (Mesocosm Gmbh) were planted in sediment filled rectangular polyethylene plastic containers (32x21x43.2 cm) to establish an in-house culture. The sediment consisted of a layer of growing medium t (DCM, vegetal raw materials, and rock phosphates) and OECD sediment (OECD GD 219 (Organisation for Economic Co-operation and Development, 2004). Following the planting of shoots, the container was filled with Smart and Barko medium (Michael Smart & Barko, 1985). The culture was covered with a clear acrylic sheet and aerated to minimize algal growth. It was kept in at 20±2°C in a climate room under white light (140±20 μmol photons/m^{-2} s^{-1}) and under a daily photoperiod of 16 h.

Single compound and mixture experiments with M. spicatum

The effects of a binary mixture of herbicide with different modes of action were investigated according to OECD TG 239 (Organisation for Economic Co-operation and Development, 2014), with deviations in the number of replicates in the treatment groups (three instead of four) and the number of shoots assessed per pot (two instead of three). The single compound experiments were conducted in parallel with the binary mixture. We selected the target concentration range and a spacing factor of 1.8 according to peer-reviewed data on *M. spicatum* from EFSA publications, Renewal Assessment Reports, and our range-finding tests (EFSA Panel on Plant Protection Products and their Residues (PPR), 2016; United Kingdom, 2018).

The test compounds diflufenican (≥ 95.0% purity) and iodosulfuron-methyl-sodium (≥98.0 %) were obtained from Sigma-Aldrich (Steinheim, Germany). They were tested as active substances (a.s.) using a solvent and not as a product, thus without any co-formulant.

In selecting the environmentally relevant toxic unit ratios (TU) of the compounds we used concentrations in edge-of-field surface water (PEC_{sw} expressed as µg a.s./L) from FOCUS Step 3 simulations (Supplementary information). Toxic unit is the ratio of the PECsw and EC50 from data available to the public (EFSA Panel on Plant Protection Products and their Residues (PPR), 2016; United Kingdom, 2018). The model calculates and reports momentary concentrations and time-weighted average concentrations in surface water and sediment that vary both temporally and spatially as a consequence of the pattern of use of a pesticide and the geo-climatic condition. The scenarios present a set of realistic worst-case representative of a range of European agricultural environments and crops (FOCUS 2012).

The derived PECsw were converted into toxic units using the data from EFSA Scientific Reports (EFSA Panel on Plant Protection Products and their Residues (PPR), 2008, 2016) on the most sensitive (EC_{50}) endpoints of the compounds to *M. spicatum.* **Error! Reference source not found.**Figure 1 visualizes the spread of the mixture ratios calculated from TU, those greater than 10 were removed to fit the plots properly in the graph and since the PECsw values were more than a magnitude lower than the respective NOEC (No Observed Effect Concentration). The concentration of the compounds varied across different FOCUS scenarios (14 Step 3 scenarios) and over time (1 to 100 d), with diflufenican:iodosulfuron-methyl sodium TU median ratio of 0.28 (n=126).

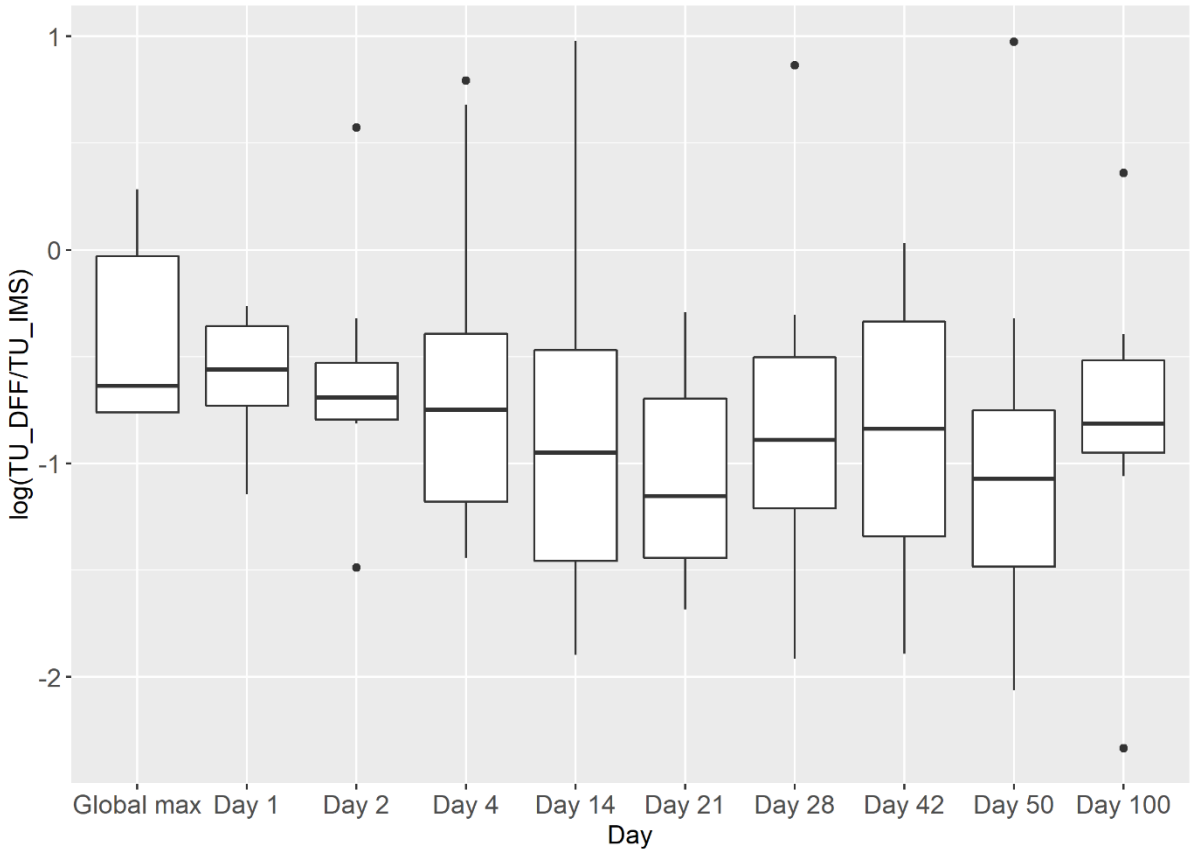


Figure 1. Mixture ratios of binary herbicide mixtures based on FOCUS edge-of-field surface water scenarios expressed as toxic units. The boxplots correspond to the spread concentrations at different days across different scenario. Median values are in the middle line, bottom and top of the boxplots give the 25th and 75th percentile. Bottom and top of the

whiskers represent the 5th and 95th percentile. The first box is based on global maximum concentrations. Global maximum concentrations (peak) can occur at a time of spray drift event, drainage event or run-off event. The succeeding boxes are based on momentary concentrations on Day 1, 2, 4, 7, 14, 21, 28, 42, 50 and 100 after the peak. DFF = diflufenican; IMS = iodosulfuron-methyl-sodium.

The experiment was performed with eight test concentration levels, Figure 2 and Supplementary Information (Table 2), each for the single and binary mixture. The stock solutions were prepared with acetone (ACS reagent, ≥ 99.5%) as a solvent since the two substances have low solubility in water. There were three replicates per test concentration, and six replicates for each medium and solvent control, in total 84 experimental units. An experimental unit (replicate) consisted of one pot (250 mL WECK jar) with a diameter of 10 cm and a height of 7.5 cm containing three shoots planted in sediment. The sediment was prepared as described in the OECD TG 219 (Organisation for Economic Co-operation and Development, 2004). The planted pot was submerged in another vessel (1.5 L WECK jar), with a diameter of 10.8 cm and a height of 20.32 cm, filled with 1.2 L of Smart and Barko medium. The shoots were allowed to establish and develop roots for at least seven days (establishment phase). At the start of the exposure phase (day 0), the medium was renewed. Deionised water (100 µL) and acetone (100 µL) were added to the medium control and solvent control groups, respectively. The stock solutions of the test substances (100 µL) were added to their respective single compound and mixture treatment groups. The final acetone concentration was 0.008% v/v. The exposure period was 14 days. Light intensity, temperature, pH, and dissolved oxygen in the test vessels were monitored throughout the experiment.

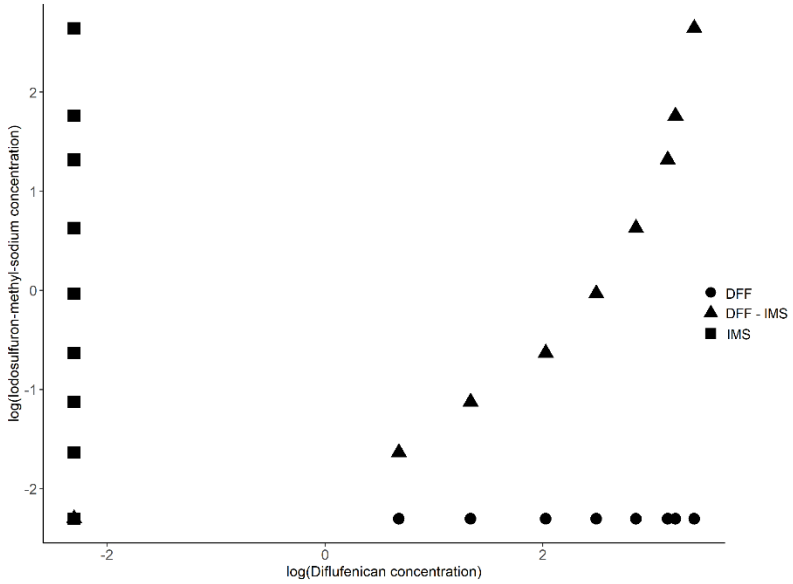


Figure 2. Test design for the binary mixture herbicide toxicity based on time-weighted average measured concentrations. Filled diamond indicates individual herbicide toxicity treatments, empty square symbols indicate mixture treatments.

We measured the fresh and dry weight for shoots and roots, total shoot length, and the number of side shoots at the end of the exposure phase. At the start of the test these were measured from the six surplus replicates. The response variable average specific growth rate were calculated for each replicate of each control and treatment group. Visual assessments were also made at the end of the exposure phase, such as the presence or absence of chlorosis.

At the end of the experiment, two of the three shoots were used for growth assessment while the third shoot selected randomly was collected and stored at -20°C for future research. For all the measurements we took the mean of the two shoots.

Analytical chemistry

Samples from the test solutions were analyzed to determine actual levels of the compounds at the start of the exposure period (time 0 days) and the end of exposure (after 14 days). Samples from replicates within each treatment concentration and control groups were pooled for analyses. The samples were analyzed by LC-MS-MS (Liquid Chromatography-Tandem Mass Spectrometry). The HPLC-instrument (Waters ACQUITY UPLC) was equipped with a Waters HSS T3 (1.8 µm) column and 2 mobile phases used in a gradient. Mobile phase A consisted of water with 10 mM ammoniumacetate, mobile phase B consisted of acetonitrile. The gradient for the first 0.25 minutes was2% B, whereas the gradient of B was increased to 98% in the first 7 minutes and held for 1 minute. After this, the linear gradient of B was reduced to 2% in one minute and this was held for another minute.

The injection volume of the HPLC-instrument was 10 µl, the oven-temperature is kept at 40°C. The detector consisted of a Triple Quadrupole mass spectrometer (without spectrometer) with an elektrospray ionisation interface that was kept at 5000 V and 500 °C. For scantype, the MRM-mode was used (Multiple Reaction Monotoring Mode) and the used collision gas was argon.

Data Analysis

Dose-response analysis

Dose-response analyses were performed on relative average specific growth rate data (i.e. growth rate was expressed relative to the average control growth rate). The average specific growth rate and relative growth rate were calculated based on Equations 1 and 2, respectively. The data from the medium and solvent controls were pooled as there was no significant effect from the use of the solvent.

$$\mu_{i-j} = \frac{\left(\ln(N_j) - \ln(N_i)\right)}{t} \quad \textbf{(1)}$$

where μ_{i-j} is the average specific growth rate from the time *i* to *j* (day^{-1}) and N_i is the measurement variable in the test or control vessel at the time *i;* N_j is the measurement variable in the test or control vessel at time *j*; and *t* is the period from *i* to *j* (days)

$$RGR_t = \frac{\mu_t}{\mu_c} \quad \textbf{(2)}$$

where RGR_t is the relative specific growth rate of the treatment *t*; μ_t is the average specific growth rate of treatment *t;* and μ_c is the average specific growth rate of control.

A three parameter log-logistic, Equation 3, dose-response model was used .

$$y = \frac{d}{1 + \left(\frac{x}{e}\right)^{\beta}} \quad \textbf{(3)}$$

where y is the relative specific growth rate of the treatment; x is the herbicide concentration (µg a.s./L); e is the single herbicide EC50 (µg a.s./L), and β is the slope parameter, *d* is the control growth rate (RGR). This analysis was done in R 3.6.2 (R Development Core Team, 2011) using the package "drc".

Mixture toxicity according to Jonker et al.

The analysis of the global interactive effects was performed in three steps adapting the methods from Nys et al. and Deruytter et al. (Deruytter et al., 2017; Hochmuth et al., 2014; Nys et al., 2015). First, the e (µg a.s./L) and β parameters of the individual compounds were used as starting values for estimating the mixture toxicity using CA (Equation 4) and IA (Equation 5), the analysis was done in R,

$$1 = \frac{x_{DFF}}{EC50_{DFF} \times \left(\frac{100-y}{y}\right)^{\frac{1}{\beta_{DFF}}}} + \frac{x_{ISM}}{EC50_{ISM} \times \left(\frac{100-y}{y}\right)^{\frac{1}{\beta_{ISM}}}} \quad \textbf{(4)}$$

$$y = 100 \times \left(\frac{1}{1+\left(\frac{x_{DFF}}{EC50_{DFF}}\right)^{\beta_{DFF}}}\right)\left(\frac{1}{1+\left(\frac{x_{ISM}}{EC50_{ISM}}\right)^{\beta_{ISM}}}\right) \quad \textbf{(5)}$$

where y is the relative specific growth rate of the treatment; x is the herbicide concentration (µg a.s./L); EC50 is the single herbicide EC50 (µg a.s./L), and β is the slope parameter; DFF is diflufenican, and ISM is iodosulfuron-methyl sodium.

Second, adapting the approach of Deruytter et al. (Deruytter et al., 2017), the CA and IA model were fitted to the whole dataset (single and mixed exposures) for each growth endpoint (Jonker et al., 2005), as a function of the toxic units of the mixture compounds. Third, in order to determine synergism or antagonism, we extended the reference models for CA and IA by introducing parameter a following the method of Jonker et al. (Jonker et al., 2005) and Haas et al. (Haas et al., 1996), the equations can be found in the Supplemental Data, and fitted the extended CA and IA model to the whole dataset. Antagonism may be concluded if parameter a is positive and synergism if it is negative.

The optimal parameters were estimated for all models with the simulated annealing optimization algorithm in R 3.6.2 (R Development Core Team, 2011) using the package "GenSA". This was used to implement a function that searches for a global minimum of a

very complex non-linear objective function with a very large number of optima (Gubian et al., 2013).

Overall model fits were compared, CA with Synergism/Antagonism (S/A) or IA with S/A, using the Akaike Information Criterion (AIC) corrected for the sample size. The improved fit is quantified by a lower SSE value of the S/S compared to CA or IA after fitting the extended model. To test the significance, a pairwise comparison between CA or IA and the S/A model, through the likelihood ratio test χ^2, was derived at degrees of freedom equal to the difference in the number of parameters in the two models, in this case, df equals 1.

Mixture toxicity according to EFSA

Risk assessment for plant protection products (PPP) follows the requirements outlined in the guidance document developed by the European Food Safety Authority (EFSA) (EFSA Panel on Plant Protection Products and their Residues (PPR), 2013b). This covers mixture risk assessment for PPP which contains more than one compound or active substance (AS). The scheme uses existing endpoints from individual AS and PPP as input variables in the prediction of mixture toxicity. We applied the principles in the mixture and risk assessment according to the rules laid out by EFSA, this is illustrated in Figure 3. Assessment was done based on individual active substance endpoints derived from this study and from the EFSA List of Endpoints for iodosulfuron-methyl-sodium and Registration Assessment Report for diflufenican (EFSA Panel on Plant Protection Products and their Residues (PPR), 2016; United Kingdom, 2018). It should be noted that the data publicly available for iodosulfuron-methyl-sodium was based on yield. In order to determine if there is an interaction of the compounds, a comparison of the predicted $EC50_{mix\text{-}CA}$ (Equation 7) for the mixture composition of the two compounds versus the measured $EC50_{mix\text{-}obs}$, expressed as (µg sum of

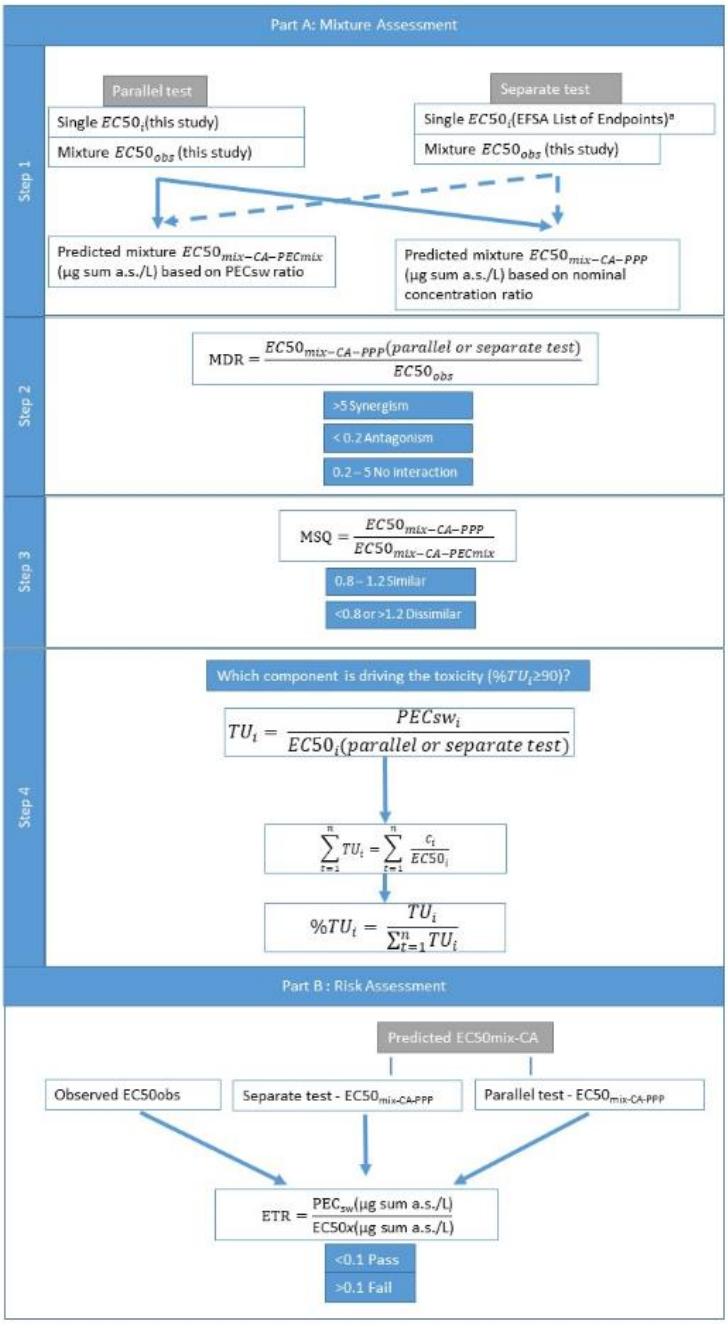
a.s./L) endpoints was done, which is referred to as Model Deviation Ratio (MDR), a method initially described by Belden et al (Belden et al., 2007). The MDR categories set by EFSA (2013) are synergistic (MDR>5), additive / not interactive (0.2≤MDR≤5), or antagonistic (MDR<0.2).

$$EC50_{mix-CA} = \left(\sum_{i=1}^{n} \frac{p_i}{EC50_i}\right)^{-1} \quad \textbf{(4)}$$

where n is the number of mixture compounds; i index from 1…n mixture compounds; p_i the i^{th} compound as a relative fraction of the mixture composition based on nominal concentrations ($\Sigma p_i = 1$); $EC50_i$ is the effect concentration (µg a.s./L); of compound, i provoking 50% effect.

The measured mixture toxicity, $EC50_{obs}$, is an endpoint normally derived from an experiment with the PPP which could contain inerts and co-formulants and is dependent on the manufacturer, in the present study we tested a mixture of the compounds without any co-formulants.

Using the PECsw values (µg a.s./L) from the FOCUS Step 3 modeling, we calculated the predicted mixture toxicity, $EC50_{mix\text{-}CA\text{-}PECmix,}$ for each FOCUS scenario and compared it with the $EC50_{mix\text{-}CA\text{-}PPP}$, labeled as Mixture Similarity Quotient (MSQ). If the mixture in the environment is different from the mixture in the PPP, 0.8<MSQ<1.2, then only the ECxmix-CA can be used in the risk assessment. The guidance document provides a decision tree on when it is required to use $EC50_{mix\text{-}CA}$ or $EC50_{mix\text{-}obs}$, for this study we decided to perform the risk assessment on both endpoints.



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295 Figure 3. General scheme of the mixture and risk assessment adapted from the EFSA aquatic
296 guidance document (EFSA 2009). MSQ : Mixture Similarity Quotient; MDR : Model
297 Deviation Ratio; ETR : Exposure Toxicity Ratio; N: number of mixture compounds; i: index
298 from 1…n mixture compounds; c_i: the concentration (µg a.s./L) of the ith compound in the
299 mixture (PPP or PECmix), e.g. concentration of the individual compound; $EC50_i$:

concentration (µg a.s./L) of compound i provoking 50% effect; PEC: Predicted Environmental Concentration in surface water.

Mixture toxicity according to Nys et al.

A method developed by Nys et al (Nys et al., 2017) to investigate the protectiveness of CA at low effect concentrations was the third method applied in this study. The degree of deviation from the CA model was done by calculating $EC10_{\Sigma TUEC10}$ for all mixture compounds at exactly 10% effect. If the mixture effect at the EC10 level follows the CA model, $EC10_{\Sigma TUEC10}$ should be equal to 1. A value smaller than one indicates a trend toward synergistic interactions relative to the CA model, while values higher than 1 suggest a trend toward antagonistic interactions. There is, however, a drawback in this method for testing with aquatic or terrestrial plants. For plants, sub-lethal endpoints are used, giving more inherent variability compared to mortality or immobility. There is a high level of uncertainty (wide confidence intervals) on the EC10 values which is largely due to the variability of responses between and within replicates. Thus, the outcome from this assessment would need to considered carefully (Staveley et al., 2018).

RESULTS

Analytical chemistry

The analysis of the test solutions revealed that at the end of the exposure period, the mean measured concentrations ranged between 45 – 117% and 42 – 77% of the initial measured concentrations for diflufenican in single and mixture tests, respectively. For iodosulfuron-methyl sodium, the mean measured concentrations ranged between 40.90 – 103% and 55 – 97% of the initial measured concentrations in single and mixture tests, respectively, Table 1. The measured values are provided in the Supplementary data.

Table 1. Time-weighted average measured concentrations of diflufenican and iodosulfuron-methyl-sodium

Nominal concentrations (µg/L)		Time weighted average measured concentrations (µg/L)		
Diflufenican	Iodosulfuron-methyl-sodium	Diflufenican	Iodosulfuron-methyl-sodium	Ratio
2.87	0.25	1.97	0.19	10.11
5.17	0.44	3.80	0.32	11.71
9.30	0.79	7.58	0.53	14.26
16.75	1.43	12.04	0.97	12.45
30.15	2.57	17.35	1.87	9.25
54.26	4.63	23.20	3.73	6.21
97.67	8.33	24.93	5.81	4.29
175.81	15	29.58	14.08	2.10

The target ratio of 12 (92% DFF and 8% IMS) in the mixture experiment and the spacing factor between concentrations were not achieved or maintained especially at the higher concentrations (concentration levels 5 to 8). The solubility of diflufenican in aqueous test medium was low, therefore test solutions were not homogenous at the higher concentrations.

The concentrations of the compound deviated more than 20% from the initial concentrations and thus we calculated the time-weighted average measured concentrations and used this to calculate endpoints.

Variability of macrophyte endpoints

The measurements of total shoot length and total shoot fresh weight in control plants met the validity criteria for doubling during the exposure phase of the test according to OECD 239 (Organisation for Economic Co-operation and Development, 2014). The mean total shoot length in control plants increased by a factor of 2.5 and the mean total fresh weight increased by a factor of 2.5 at test termination, Day 14. The coefficient of variations of growth rate endpoints calculated for each treatment and concentration was in the range of 4% to 41%, 8% to 41%, 3% to 122%, 0 to 99 %, for fresh weight shoots, total shoot length, dry weight shoots, and number of side shoots, respectively. The coefficient of variations of growth rate endpoints calculated for the control was 232%, 50%, 16%, and 41% for fresh weight shoots, total shoot length, dry weight shoots, and number of side shoots, respectively. For the yield of fresh weight shoots the coefficient of variation was 38%, which is slightly above the trigger of 35% according to the validity criteria.

Individual compound dose-response analysis

The single herbicide growth rate EC50 values and slopes are listed in Table 2 for each of the growth endpoints. Based on the results, iodosulfuron-methyl sodium was about one order of magnitude more toxic than diflufenican.

The concentration-response curves are shown in Figure 4.

Table 2. The log-logistic concentration response curve parameter estimates (EC10, EC20, EC50 and slope) based on measured concentrations of diflufenican and iodosulfuron-methyl sodium and relative growth rate.

Treatments	EC10±SE (µg a.s./L)	EC20±SE (µg a.s./L)	EC50±SE (µg a.s./L)	β±SE
Diflufenican				
Fresh weight shoots	0.58±1.50 (n.c.)	1.75±1.50 (n.c.)	11.64±3.64 (3.00 – 20.25)	0.73±0.29 (0.03 – 1.43)
Total shoot length	0.22±0.89 (n.c.)	1.56±3.93 (n.c.)	45.20±55.43 (n.c.)	0.41±0.35 (n.c.)
No. of side shoots	1.91±0.78 (0.07 – 3.76)	4.06±1.12 (1.42 – 6.70)	14.70±1.79 (10.83 – 19.30)	1.08±0.20 (0.61 – 1.59)
Iodosulfuron-methyl sodium				
Fresh weight shoots	0.15±0.06 (0.01 – 0.30)	0.30±0.09 (0.10 – 0.50)	1.05±0.17 (0.64 – 1.47	1.11±0.19 (0.67 – 1.55)
Total shoot length	0.59±0.11 (0.33 – 0.86)	0.72±0.09 (0.49 – 0.95)	1.00±0.06 (0.85 – 1.14)	4.22±1.22 (1.23 – 7.21)
No. of side shoots	0.19±0.05 (0.08 – 0.30)	0.31±0.05 (0.18 – 0.44)	0.74±0.07 (0.58 – 0.92)	1.61±0.28 (0.98 – 2.31)
Mixture*				
Fresh weight shoots	7.80±4.30 (n.c.)	11.52±4.61 (0.23 – 22.81)	22.50±4.04 (12.60 – 32.40)	2.07±0.83 (0.02 – 4.12)
Total shoot length	5.00±1.36 (1.70 – 8.32)	6.91±1.45 (3.37 – 10.46)	12.00±1.43 (8.50 – 15.50)	2.51±0.52 (1.25 – 3.78)
No. of side shoots	0.81±0.32 (0.02 – 1.60)	1.70±0.52 (0.43 – 2.97)	6.03±1.08 (3.39 – 8.67)	1.09±0.16 (0.71 – 1.48)

SE = standard error

The 95% confidence interval (lower – upper) are reported in parentheses.

n.c. not possible to calculate the 95% confidence interval or negative values are derived

*Concentration expressed as sum of the active substances

For the endpoints fresh and dry weight roots, we could not fit the 3-parameter log-logistic to the data as there was no clear dose (monotone) response for the single compounds, for both diflufenican and iodosulfuron-methyl sodium, and the mixture. For dry weight shoots, there was no clear dose-response for iodosulfuron-methyl sodium and the mixture experiments and no positive confidence intervals could be calculated. These growth endpoints were no longer used in the succeeding assessment. It should be noted that assessment of roots has been known to be practically challenging because of the miniature

size of the roots, while we attempted our best effort to collect this data by thoroughly washing off the sediment from the roots we judged the data to be unreliable for further assessment.

At the end of the experiment, the shoots in the diflufenican and mixture treatment groups showed chlorosis (pinkish color), this was seen across all treatment levels.

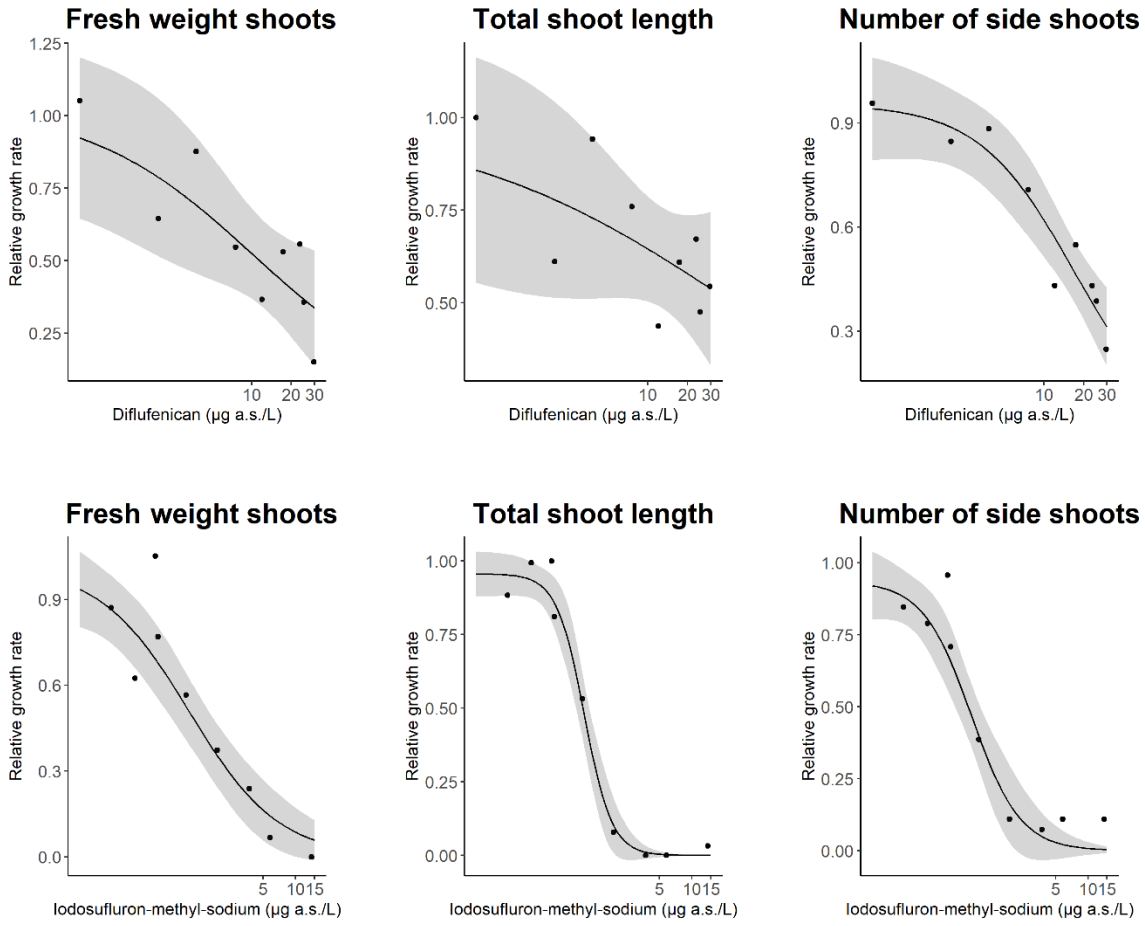

Figure 4. Single compound dose response curves of the relative growth rates for the growth parameter fresh weight shoots, total shoot length and number of side shoots. Each dot represents observed values based on average data (*n*=3) for each treatment level and the confidence intervals are represented by the shaded region.

Mixture assessment according to significance testing - Jonker et al. 2005

The predicted relative growth rate based on CA and IA for the binary herbicide mixture treatments is shown as a function of the sum of toxic units (TU) in Figure 5.

Comparing the effect of diflufenican and iodosulfuron-methyl sodium on the growth rate of *M. spicatum* to CA resulted in an SSE in the range of 0.13 – 0.23 for the different growth endpoints. Adding parameter a to the CA model decreased the SSE significantly ($p[\chi^2]$<0.0001, which could indicates interaction. For all growth endpoints, except the number of side shoots, parameter $a > 0$ which could indicate decreased toxicity (antagonism) relative to CA, Table 3. For the number of side shoots the value of a is -0.21 which is close to the trigger, parameter $a < 0$, indicating slight synergism. In Figure 5, the curve of the mixture is slightly below the predicted toxicity curves at lower toxic units.

Table 3. The optimal parameter values $\boldsymbol{a}$ according to the method proposed by Jonker et al. and the corresponding SSE (sum of squares error) for each growth endpoint.; CA = concentration addition; IA = independent action; S/A = synergistic or antagonistic deviations.

Growth parameter	Sum of Squares Error		Parameter	Sum of Squares Error		Parameter
	CA	S/A	$\boldsymbol{a}$	IA	S/A	$\boldsymbol{a}$
Fresh weight shoots	1.45	0.23	7.42	0.95	0.23	5.64
Total shoot length	0.97	0.2	2.43	0.23	0.17	2.98
Number of side shoots	0.85	0.13	-0.21	0.17	0.16	0.28

When comparing the same data to IA, this resulted in an SSE in the range of 0.16 – 0.23 for the different growth endpoints. Adding parameter a to the IA model to describe synergism/antagonism decreased the SSE significantly ($p[\chi^2]$<0.05) for fresh weight and total shoot length, which indicates interaction but not for the number of side shoots($p[\chi^2]$=0.65). For all growth endpoints, parameter $a > 0$, indicating decreased toxicity (antagonism) relative to independent action except for number of side shoots.

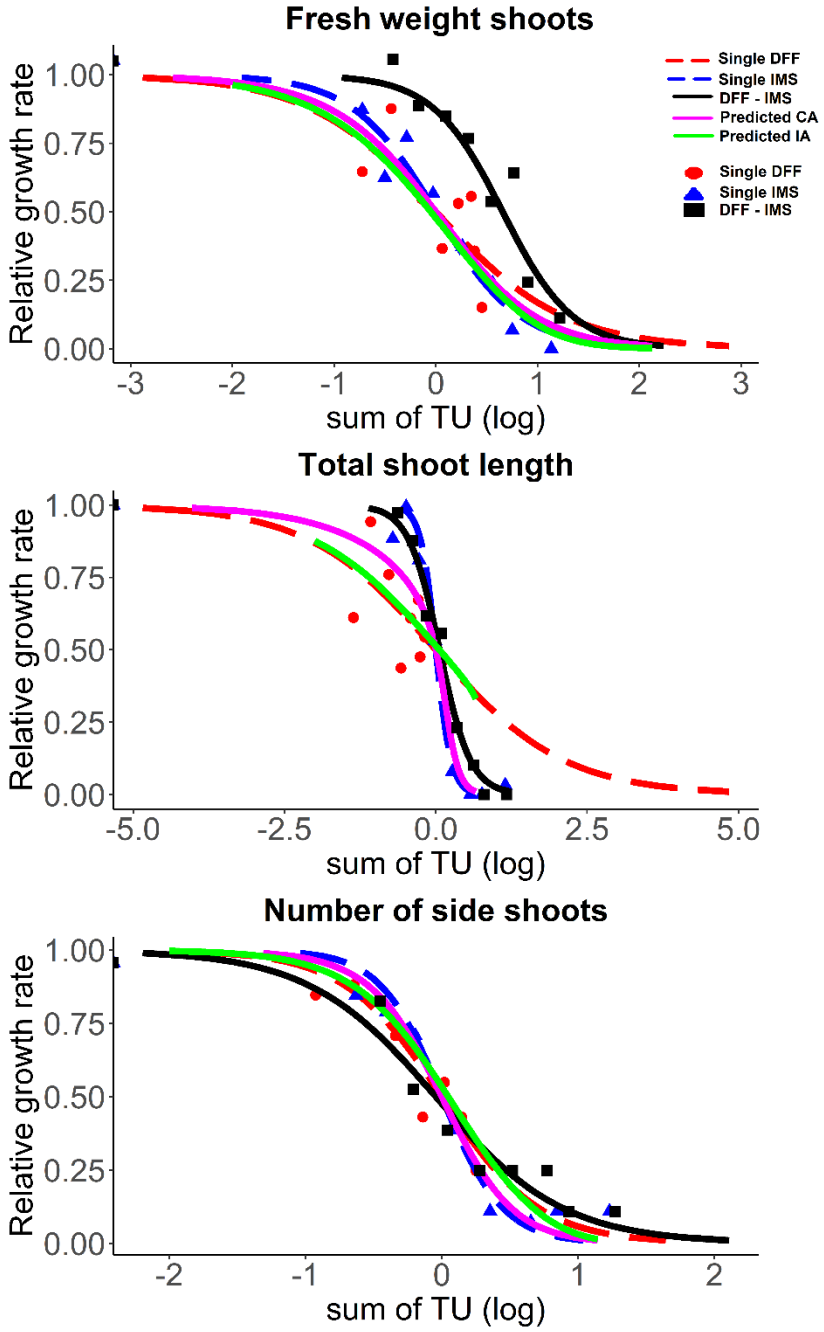

Figure 5. Observed and predicted relative growth rates in the mixture combination of diflufenican and iodosulfuron-methyl-sodium as a function of the sum of the toxic units based on their time weighted concentrations. Plots could not be generated with dry weight shoots due to the lack of clear monotone dose response. The black solid line (—) is the

mixture dose-response curve. The coloured solid lines are the predictions of concentration addition (—) and independent action (—). The long dashed lines are the single compound dose-response curves: DFF (— —) and IMS (— —). Each marker (● = DFF mixture; ▲ = IMS; ■ = DFF-IMS) represents observed values based on average data (*n*=3).

Mixture assessment according to EFSA 2013

The measured mixture toxicity values, EC50obs, presented in Table 2, are based on the experiment performed with the binary mixture of diflufenican and iodosulfuron-methyl sodium. The EC50 is expressed based on the sum of the time-weighted average concentrations of the two compounds. The predicted mixture toxicity assumed CA and used the target DFF/IMS concentration ratio of 12 (92% DFF and 8% IMS), this is to align with the method used in evaluating PPP mixtures where the nominal content of the compounds are used. The target ratio was not maintained or achieved at all treatment levels and this could affect the outcome of the assessment. It should, however, be underlined that pesticide mixture risk assessments are not uncommonly performed with studies where the measured concentrations deviate more than 20% from the applied or initial concentrations.

Concentration addition holds for the mixture in all growth endpoints as the MDR values are between 0.2 and 5, for both parallel and separate tests, respectively, Table 4.

Table 4. Comparison of predicted and measured mixture toxicity using the Model Deviation Ratio (MDR) principle. $EC50_{mix\text{-}CA(pred)}$ is based on Equation 7; $ErC50_{obs}$ is based on observed or measured. The effect concentrations are expressed as µg sum a.s./L.

		Parallel test				Separate test			
Growth endpoints	$EC50_{obs}$	EC50 DFF (µg a.s./L)	EC50 IMS (µg a.s./L)	$EC50_{mix\text{-}CA\text{-}PPP}$ (µg a.s./L)	MDR	EC50 DFF_a (µg a.s./L)	EC50 IMS_b (µg a.s./L)	$EC50_{mix\text{-}CA\text{-}PPP}$ (µg a.s./L)	MDR
Fresh weight shoots	22.50	11.64	1.05	5.86	0.29	101	2.51	24.73	1.24
Total shoot length	12.60	45.20	1.00	10.10	0.84	101	2.03	20.90	1.64
No. of side shoots	6.03	14.70	0.74	5.92	0.99	n.d.			

n.d. = not determined

a = European Commission (Renewal Assessment Report 2018, List of Endpoints, published for public consultation (Kuhl, K. 2016a, Report no. EBDCN163, Bayer CropScience)

[b] = EFSA Panel on Plant Protection Products and their Residues (PPR). (2016). Peer review of the pesticide risk assessment of the active substance iodosulfuron-methyl-sodium (approved as iodosulfuron), List of Endpoints; Summary of ecotoxicological studies for iodosulfuron-methyl, 2014-04-07, Banman C.S, Matlock D, and Lam C.V., 2012. Report no. EBIML032, Bayer CropScience)

We calculated the $EC50_{mix\text{-}CA\text{-}PECmix}$ using endpoints from the parallel test (this study) and separate test (EFSA List of Endpoints). The mixture composition tested in this study appears to be similar to the composition of several mixture FOCUS scenarios in the environment (MSQ between 0.73 – 3.91, Supporting Information, Table 8). This is expected as we used the DFF:IMS TU median ratio in establishing the test concentrations, where the TU is calculated with the FOCUS Step 3 values. This is true only when endpoints from parallel tests are used. However, it should be noted that the FOCUS mixture composition used in calculating the MSQ according to the EFSA Guidance was based on PECsw maximum values, although it is likely that the ratio of the two active substances could change over time.

The final step in the mixture assessment is to determine if one mixture compound drives ($\%TU_i \geq 90\%$) the toxicity. For the growth endpoints fresh shoot weight and total shoot length, iodosulfuron-methyl sodium drives the toxicity in four scenarios (D2_ditch, D2_stream, R1_stream, and R4_stream), detailed results are provided in the Supporting Information.

Finally, in the risk assessment, we compared the outcome when using endpoints from parallel and separate tests. The risk is unacceptable in the majority of the FOCUS scenarios for the predicted mixture toxicity endpoints, while it is acceptable when the endpoint used is based on observed mixture toxicity, EC50obs.

Mixture assessment according to Nys et al.

According to the third method, the MIF for the growth endpointsnumber of side shoots and total shoot length is less than one, 0.4 and 0.5, respectively. which indicates synergistic interactions while antagonistic interaction can be concluded for fresh weight, Table 5.

Table 5. Observed mixture effects as a function of sum of toxic units, expressed relative to EC10. $EC10_{\sum TUEC10}$ = Mixture interaction Factor(MIF)

Growth endpoints	Best fitting model	$EC10_{\Sigma TUEC10}$ (MIF)	Standard error	Parameters	
				b	e
Fresh weight shoots	LL.3	5.40	2.52	0.95	55.01
Total shoot length	LL.3	0.53	0.19	1.46	2.40
No. of side shoots	LL.3	0.36	0.26	0.85	4.71

A comparison of the mixture assessment from the different methods is outlined in Table 6. It shows the difference in the conclusion depending on the method used and the corresponding effect level. For fresh weight shoots and number of side shoots, the conclusion is consistent between the Jonker et al. and MIF method. For total shoot length, synergism relative to CA was concluded based on the MIF method.

Table 6. Deviations from concentration addition according to different evaluation methods

	Method/Effect level		
	MDR_{PPP}	Jonker et al. 2005	MIF – Nys et al. 2017
Growth endpoints	EC50	EC50	EC10
Fresh weight shoots	CA-no interaction	CA-antagonism	CA-antagonism
Total shoot length	CA-no interaction	CA-antagonism	CA-synergism
Number of side shoots	not determined	CA-synergism	CA-synergism

MDR_{PPP}: predicted mixture toxicity based on the mixture composition

DISCUSSION

Aquatic plants exhibit diversity in their photosynthetic mechanism (Van et al., 1976), and differ from terrestrial plants such that they cannot be easily classified as either C3 or C4 plants. The *M. spicatum*, for example, exhibits characteristics of both C3 and C4 plants. When testing the toxicity of herbicides it is important to understand the mechanism in which they affect the plant at the biochemical level and also consider the multiple pathways that induce the phenomenological effect. Toxicity assays on aquatic plants are limited to the measurement of growth which is affected by numerous biosynthetic pathways.

Diflufenican inhibits carotenoid biosynthesis, although the exact target site is unknown there is evidence on the accumulation of carotenoid precursors such as phytoene suggesting that the herbicide inhibits a reaction in a sequence between phytoene and lycopene and the likely target is phytoene desaturase (Ashton et al., 1992; Ridley, 1982; Sandmann, 1987; Sandmann et al., 1984). Carotenoids are integral in building the photosynthetic apparatus of plants. When it is inhibited the photosynthetic efficiency decreases. Iodosulfuron-methyl-sodium inhibits acetolactate synthase which is essential in the biosynthesis of branched amino acids: valine, leucine, and isoleucine. These amino acids are responsible for elongation and cell division.

Diflufenican manifests chlorosis in plants, which was also observed in the present study, and inhibition of growth. The most sensitive growth rendpoints we determined were those related to biomass such as dry and fresh weight of the shoots (Table 2).

For iodosulfuron-methyl sodium, its mode of action suggests an effect which is targeted on the development of new shoots which ultimately relates to inhibition of growth. All growth endpoints have comparable sensitivity to iodosulfuron-methyl sodium except for dry weight shoots where no clear dose-response was observed, furthermore the estimates

were unreliable due to the negative confidence intervals derived (Table 2). Several studies have also reported no effects on dry weight shoots of aquatic plants when exposed to sulfonylurea herbicide (Nuttens et al., 2016; Wendt-Rasch et al., 2003; Wieczorek et al., 2017)

In the present study, we investigated the interactive effects of the binary mixture of diflufenican and iodosulfuron-methyl-sodium on *M. spicatum*. We evaluated the deviation from CA based on: significance testing (Jonker et al., 2005), the MDR method (EFSA Panel on Plant Protection Products and their Residues (PPR), 2014), and MIF (Nys et al., 2017), and deviation from IA based solely on significance testing. Most of the growth endpoints measured in binary mixture demonstrate that diflufenican and iodosulfuron-methyl acted antagonistically relative to either CA or IA model. Our results support the findings of Cedergreen et al. (Cedergreen et al., 2007) on *Pseudokirchneriella subcapitata*, selected terrestrial plants, and *Lemna minor* that for herbicides with different modes of action synergism was not observed instead approximately 70% of the mixture of herbicides exhibited significant antagonism when evaluated against both CA and IA.

The joint effect of diflufenican and iodosulfuron-methyl sodium on the number of side shoots appears to be slightly additive. These two compounds, however, have different molecular target sites and cause different toxicological responses. This observation could be explained by Abendroth et al. (Abendroth et al., 2011) who found CA as not biologically appropriate. To properly evaluate the correct mixture model, knowledge of the degree of similarity or dissimilarity of mixture components is needed. One limitation in the present study, and other existing mixture experiments on plants, is the lack of in-depth understanding of the components' modes of action.

538 The use of CA is not considered biologically appropriate for the present study. The
539 combination of carotenoid biosynthesis inhibitor and ALS inhibitor does not support the
540 assumption of dose additivity. The dose-response curves of diflufenican and iodosulfuron-
541 methyl for all growth rate endpoints lack parallelism thus the two compounds are not mere
542 dilutions of one another. It is more plausible to justify that the effect on the number of side
543 shoots was primarily driven by iodosulfuron-methyl sodium, its mode of action is to inhibit
544 the development of new shoots and also to re-allocate material inside the plant, hence affects
545 the total shoot length but not on the dry weight.
546 The conclusion regarding the presence of interaction relative to CA model varied
547 across different methods of assessment and growth endpoints. The interaction relative to IA
548 model can only be assessed via the Jonker et al. (Jonker et al., 2005) method. The MDR
549 method uses only the CA model as it is considered as the most relevant for hazard
550 characterization even for mixtures containing compounds with different modes of action. The
551 CA model is preferred for risk assessment purposes as it is the most conservative. The
552 procedure in performing mixture toxicity assessment for plant protection products is laid out
553 in the EFSA Aquatic Guidance Document (EFSA Panel on Plant Protection Products and
554 their Residues (PPR), 2013b) which utilizes existing data of individual compounds and their
555 mixture formulations. This method enables mixture toxicity assessment by simply calculating
556 the Model Deviation Ratio without the need to perform single compound and mixture
557 experiments in parallel and without statistical significance testing, as was done in the present
558 study. When calculating the MDR based on measured content of the compounds in the
559 mixture, it appears to follow CA without interaction (neither antagonistic nor synergistic) and
560 when using the method of Jonker et al. (Jonker et al., 2005) antagonism against CA and IA is
561 statistically significant. Interestingly, for fresh weight shoots the MDR value was very close
562 to the limit for antagonism, which would then be aligned with the conclusion derived

563 according to MIF (Nys et al., 2017) and Jonker et al. (Jonker et al., 2005). The use of EC10
564 has an increased uncertainty, the confidence intervals we derived were very wide and even
565 some had negative values (Staveley et al., 2018)
566 According to Coors and Frische (Coors & Frische, 2011), high(Staveley et al.,
567 2018)er frequency and a larger degree of deviation between predicted and observed in a
568 mixture toxicity assessment, similar to the EFSA Aquatic Guidance ((EFSA Panel on Plant
569 Protection Products and their Residues (PPR), 2013b)), suggests that the mixture toxicity
570 predictions established in the scientific literature are not fully aligned with the current
571 regulatory scheme. They identified that a considerable part of the uncertainty in the
572 prediction was caused by the much larger heterogeneity of the data used as input for the
573 prediction. Nonetheless, this data heterogeneity did not lead to over or under estimation of
574 mixture toxicity. This limitation of heterogeneous data has been addressed in the present
575 study, as the data for predicting mixture toxicity and assessing interactions were all derived
576 from parallel experiments of a single and binary mixture, performed at the same time and in
577 the same room. This difference in conclusion across different growth endpoints and methods
578 proves that although the use of CA model for compounds with dissimilar modes of action is
579 conservative, it, however, prevents proper interpretation of mixture effects and for aquatic
580 macrophytes it is also restricted to the growth endpoint being used.
581 Further study needs to be done on the mixture toxicity of pesticides based on relevant
582 environmental ratios. They should be analysed applying different methods of assessing
583 interaction to strengthen the protectiveness and accuracy of the existing regulatory approach.
584 It should also be explored on how to extend the work and model effects of time variable
585 exposures of pesticide mixtures to rooted macrophytes.
586
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