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Biodegradation and toxicity of a maize herbicide mixture: mesotrione, nicosulfuron and S-metolachlor

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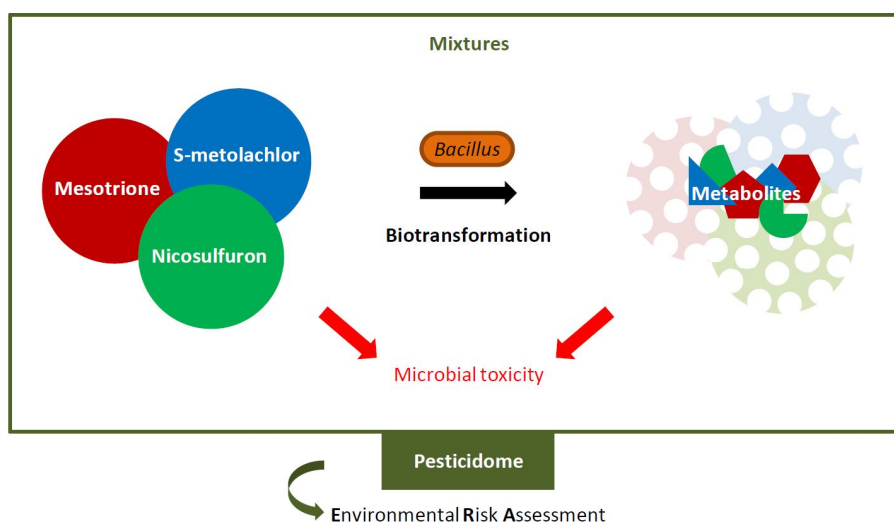
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Graphical abstract



Highlights

- *B. megaterium* transforms both mesotrione and nicosulfuron, alone or in mixture.
- Biotransformation pathways are not affected in binary and ternary mixtures.
- Nicosulfuron degradation and metabolite formation kinetics is mixture dependent.
- Binary/ternary mixtures of active ingredients show synergistic effects (Microtox[®]).
- Metabolite mixtures +/- S-metolachlor show synergism, antagonism or additivity.

Abstract

The prediction of chemical mixture toxicity is a major concern regarding unintentional mixture of pesticides from agricultural lands treated with various such compounds. We focused our work on a mixture of three herbicides commonly applied on maize crops within a fortnight, namely mesotrione (β -triketone), nicosulfuron (sulfonylurea) and S-metolachlor (chloroacetanilide). The metabolic pathways of mesotrione and nicosulfuron were qualitatively and quantitatively determined with a bacterial strain (*Bacillus megaterium* Mes11). This strain was isolated from an agricultural soil and able to biotransform both these herbicides. Although these pathways were unaffected in the case of binary or ternary

herbicide mixtures, kinetics of nicosulfuron disappearance and also of mesotrione and nicosulfuron metabolite formation was strongly modulated. The toxicity of the parent compounds and metabolites was evaluated for individual compounds and mixtures with the standardized Microtox[®] test. Synergistic interactions were evidenced for all the parent compound mixtures. Synergistic, antagonistic or additive toxicity was obtained depending on the metabolite mixture. Overall, these results emphasize the need to take into account the active ingredient and metabolites all together for the determination of environmental fate and toxicity of pesticide mixtures.

Keywords: Herbicide mixture; Simultaneous biotransformation; *Bacillus megaterium* Mes11; Microbial toxicity

1. Introduction

Agricultural practices have considerably evolved over the last few decades, adopting Integrated Pest Management. This includes various management practices to favour growth of healthy crops and minimize pesticide use. This trend has triggered use of new-generation pesticides applied at lower doses but often in mixture, in order to improve their efficiency and the range of weeds treated. These changes lead to potential contamination of surface and ground water by a variety of compounds from different sources. The prediction of the toxicity of such unintentional mixtures is a major challenge because of their complex and variable composition. However, many of the parent compounds and their metabolites are still unidentified and toxicity data are lacking [1].

The toxicity of a given mixture is predicted with mathematical models combining (eco)toxicological data of individual compounds and their chemical concentration. This approach does not consider potential synergistic effects of pesticides and metabolites mixtures

[2]. Identifying biodegradation pathways of chemicals in mixtures is therefore essential to include all relevant molecules in monitoring studies (i.e. synergistic, co-occurring and highly hazardous compounds).

We focused our work on three herbicides, commonly applied within a fortnight on maize crops: in pre-emergence S-metolachlor (chloroacetanilide) and in post-emergence, a mixture of mesotrione (β -triketone) and nicosulfuron (sulfonylurea). Mesotrione (2-(4-methylsulfonyl-2-nitrobenzoyl)cyclohexane-1,3-dione) is a selective herbicide inhibiting carotenoid biosynthesis in a wide range of broad-leaved and grass weeds. An application rate (AR) of 150 g active ingredient (a.i.)/ha is adopted [3]. Two major metabolites, AMBA (2-amino-4-methylsulfonylbenzoic acid) and MNBA (4-methylsulfonyl-2-nitrobenzoic acid), are often detected in the environment [4,5] and are more persistent in the soil than the parent compound [6]. The Microtox[®] test also showed that AMBA was more toxic than mesotrione towards *Aliivibrio fischeri* (formerly *Vibrio fischeri*) [7]. Nicosulfuron (2-[(4,6-dimethoxypyrimidin-2-ylcarbamoyl)sulfamoyl]-N,N-dimethylnicotinamide) is a selective herbicide inhibiting the branched-chain amino acid biosynthesis of annual grass, broad-leaved and perennial weeds, with an AR of 60 g a.i./ha [8]. Its major transformation pathway is the hydrolysis of the sulfonylurea bridge, leading to ADMP (2-amino-4,6-dimethoxypyrimidine) and ASDM (2-(aminosulfonyl)-N,N-dimethyl-3-pyridinecarboxamide). ADMP is more toxic towards *A. fischeri* than the parent compound [9]. S-metolachlor, the active enantiomer of (2-chloro-N-(2-ethyl-6-methylphenyl)-N-[2-methoxy-1-methylethyl]acetamide) inhibits the very long chain fatty acid biosynthesis of annual grass and broad-leaved weeds, with an AR of 1920 g a.i./ha [10]. Its major metabolites (metolachlor oxalinic acid and ethanesulfonic acid) are often detected in higher concentration in water than the parent compound [11,12].

Despite their sequential application on soil surface, these three herbicides are detected in surface and ground water, S-metolachlor being the most frequently detected [13,14]. Their

monitoring carried over the past 10 years in France revealed the combined presence of these herbicides, either in binary or ternary mixtures [13,14]. However, the combinations of these herbicides have not been studied extensively, in particular regarding their biotransformation, the predominant process involved in their environmental fate. Several isolated microbial strains, able to biotransform each herbicide separately, have already been described for mesotrione [15–20], nicosulfuron [9,21–26] and to a lesser extent for S-metolachlor [27]. Some studies have also investigated the microbial toxicity (Microtox[®]) of pure active ingredients and formulations (alone or in mixtures) of the three herbicides [7,28]. However, no data are available concerning the effect of mixing these herbicides on their biotransformation kinetics and the toxicity of parent compounds and/or metabolites.

A preliminary screening of bacterial strains for their ability to transform the three herbicides revealed that *Bacillus megaterium* Mes11 (noteworthy for its mesotrione-degrading activity [15]) was also able to transform nicosulfuron. The aims of this study were therefore to determine: (i) the biotransformation pathways of each of these three herbicides and the effects of each compound on the biotransformation of the others for this model strain and (ii) the toxicity of the parent compounds and metabolites, alone or in mixtures toward *A. fischeri* using the standardized Microtox[®] test. The present study will therefore provide relevant information on the biotransformation (e.g. identity of metabolites, biotransformation kinetics) and toxicity of the pesticide mixtures studied (parent compounds and metabolites).

2. Materials and Methods

2.1. Bacterial strain, chemicals and media

The bacterial strain *Bacillus megaterium* Mes11 was previously isolated from a maize cultivated soil [15]. Nicosulfuron (Pestanal, purity 99.6%), mesotrione (Pestanal, purity 99.9%), S-metolachlor (Pestanal, purity 98.4%), AMBA, ADMP (purity 98.0%) and Tryptic

Soy broth (TS) medium were purchased from Sigma Aldrich (France) and ASDM (purity 98%) from J and K Scientific (Germany). The composition of Glucose-mineral salt medium (GSM), pH 6.7, was previously described by Carles et al. [9].

2.2. Biotransformation assays

The strain Mes11 was grown in TS broth (750 mL) supplemented with 0.1 mM mesotrione, at 28 °C on a rotary shaker (150 rpm). The cells were harvested after 24 h of culture and centrifuged at 8,000 g for 15 min at 4 °C. The bacterial pellet was washed in NaCl (8 g/L). The resting cells were then resuspended in 150 mL of GSM supplemented with 1 mM of mesotrione, nicosulfuron and S-metolachlor, either alone, in binary or ternary mixtures. The same media without inoculation served as abiotic controls. Each condition was carried out in triplicate. The cultures were incubated in the dark at 28 °C under agitation at 150 rpm and sampled periodically (3 x 1 mL). After centrifugation, the supernatants were frozen at -25 °C until analysis by HPLC, ¹H NMR and LC/ESI-MS.

One millilitre of Mes11 culture was used to extract parent compounds/metabolites adsorbed on biomass. After centrifugation (13,000 g, 5 min), the pellet was suspended in 500 µL absolute ethanol (purity > 99.5%, v/v). The suspension was stirred overnight at room temperature and centrifuged (13,000 g, 5 min). The extraction was performed twice.

2.3. Analysis of the herbicides and their metabolites

2.3.1. Analysis by HPLC

The herbicide analyses were monitored by HPLC on an Agilent Series 1100 chromatograph, equipped with a Diode Array Detector (DAD) and a reverse phase column (Zorbax Eclipse XDB-C18, 3.5 µm, 75 mm x 4.6 mm) at 22 °C. The mobile phase was composed of acetonitrile (Solvent A) and acidified water (H₃PO₄, 0.01% v/v; pH 2.9)

(Solvent B) used at a flow rate of 1 mL/min and with different linear gradients according to the herbicide analysed (Table S1). Injection volume: 5 μ L. Each sample was injected twice. The measure of precision was less than 2%. The limits of quantification and detection of the different molecules tested are given in the Table S2.

2.3.2. Analysis by ^1H NMR

^1H Nuclear Magnetic Resonance (NMR) analyses were performed at 25 $^\circ\text{C}$ at 500.134 MHz on an Avance 500 Bruker spectrometer (Bruker Biospin, Wissembourg, France) equipped with a triple-resonance (^1H , ^{13}C , ^{15}N) inverse probe with 5-mm-diameter tubes containing 600 μL of sample (540 μL of supernatant with 60 μL of a 5 mM of tetra-deuterated sodium trimethylsilylpropionate solution in D_2O), with water being suppressed by a classical two phase-shifted pulse saturation sequence (double pulsed field gradient echo sequence WATERGATE) according to Durand et al. [16].

2.3.3. Analysis by LC-MS

LC/ESI-MS analyses were performed on a ThermoScientific UHPLC Ultimate 3000 RSLC coupled with an Orbitrap Q-Exactive analyser. The crude supernatants were harvested (13,000 g, 5 min) before LC-MS analyses and directly injected without any further treatment. The analyses were carried out in positive mode. The UHPLC was equipped with a Kinetex EVO C18; 100 x 2.1 mm; 1.7 μm (Phenomenex) at 30 $^\circ\text{C}$ with a gradient acetonitrile + 0.1% formic acid (Solvent A) and water + 0.1% formic acid (Solvent B): 0–7.5 min: 5–99% A (linear); 7.5– 8.5 min: 99% A; 8.5–9 min: 99–5% A; 9–11 min: 5% A. Flow: 0.45 mL/min. For the mass spectrometer, gaseous N_2 was used as nebulizer gas (50 L/h). The spray voltage was 3.2 kV.

2.4. Modelling of the herbicide degradation and metabolite formation/disappearance kinetics

The transformation kinetics of nicosulfuron and those of mesotrione and nicosulfuron metabolites were fitted with the DFOP (Double First-Order in Parallel) model [29] using the CAKE (Computer Assisted Kinetic Evaluation) V3.2 software (Tessella Technology & Consulting, UK), characterized by the following equation:

$$C_t = C_0 g^{-k_1 t} + C_0 (1 - g)^{-k_2 t} \quad (\text{Equation 1})$$

where t is the incubation time, C_t the chemical concentration at time t , C_0 the initial concentration of the parent herbicide or the maximal concentration of the intermediate metabolites, g the fraction of C_0 applied to the first term of the equation 1 (*i.e.* the fraction of the initial chemical that degrades at the fastest rate), k_1 and k_2 the rate constants for the first and the second terms of the equation 1, respectively (in day^{-1}). The dissipation time x (DT_x) value is the time required for the concentration to decline to $x\%$ of the initial value.

The kinetics of mesotrione transformation and those of mesotrione/nicosulfuron metabolite production (intermediate metabolites and end-products) were fitted with the DoseResp function of the OriginPro V8 software (Origin Lab Corporation, USA), characterized by the following equation:

$$C_t = \frac{C_{max}}{1 + 10^{(h_{50} - t)(p)}} \quad (\text{Equation 2})$$

where t is the incubation time, C_t the chemical concentration at time t , C_{max} the maximal concentration of this chemical, h_{50} the time required for the concentration to reach/decrease by 50% of the final value and p the rate constant.

2.5. Mixture toxicity assays

2.5.1. Mixture ratio

Mixture toxicity assays were carried out in parallel to biotransformation tests. The active ingredients and/or metabolites were mixed according to the initial molar concentration of the parent compounds (starting condition) and the final molar concentration of the metabolites (after 20 days of incubation). In our study, all the ratios were 1:1 (binary mixture), 1:1:1 (ternary mixture), and 1:1:1:1 (quaternary mixture), except for mixtures containing AMBA which has a proportion of 0.31.

2.5.2. *Microtox*[®] assays

The herbicides and their metabolites were dissolved in DMSO and serial-diluted (8 solutions ranging from 0 to 800 μM). The dose response curve obtained was used to determine the median inhibitory concentration (IC_{50}) of each compound (alone or in mixture), which is the concentration required to induce a 50% decrease of *Aliivibrio fischeri* bioluminescence compared to the untreated bacteria after 15 min of incubation, as previously described [7,28,30]. The calculations to estimate the IC_{50} were carried out with the OmniTM software packages (version 1.18, AZUR Environmental Ltd.) as described by Doe et al. [31].

2.5.3. Modelling of mixture toxicity

The mixture toxicities were compared with the expected toxicity values predicted by the Concentration Addition (CA) and the Independent Action (IA) models [32]. The prediction of combined effects of mixture-components based on CA can be expressed as:

$$\text{IC}_{50, \text{mix}} = \left(\sum_{i=1}^n \frac{p_i}{\text{IC}_{50, i}} \right)^{-1} \quad (\text{Equation 3})$$

where $\text{IC}_{50, \text{mix}}$ is the total concentration of the mixture that causes 50% inhibition, p_i the proportion of the i^{th} component in the mixture, n the number of components in the mixture, and $\text{IC}_{50, i}$ the median inhibitory concentration of the i^{th} compound when applied alone.

The prediction of combined effects of mixture-components based on IA can be expressed as:

$$E(c_{mix}) = 1 - \prod_{i=1}^n (1 - E(c_i)) \quad (\text{Equation 4})$$

where $E(C_{mix})$ is the total effect of the mixture, n the number of components in the mixture and $E(C_i)$ is the fractional effect ($x\%$) caused by the i^{th} compound when applied alone. $E(C_i)$ was calculated from concentration response functions determined for each single substance. The value of $E(C_{mix})$ satisfying the equation for a given effect level x was computed by an iterative procedure using the OriginPro 8 software.

The synergy was evaluated with the ratio of predicted *versus* observed IC_{50} values, called Model Deviation Ratio (MDR) and defined as:

$$MDR = \frac{\text{Expected}}{\text{Observed}} \quad (\text{Equation 5})$$

where “*Expected*” corresponds to the $IC_{50, mix}$ predicted by the model (CA or IA), and “*Observed*” is the $IC_{50, mix}$ observed with the Microtox[®] test (*i.e.* the concentration of each component in the mixture causing 50% inhibition of *A. fischeri* bioluminescence). The compounds were then classified as synergistic ($MDR \geq 2$), additive ($0.5 < MDR < 2$), or antagonistic ($MDR \leq 0.5$).

2.6. Statistical analyses

Statistical analyses were carried out using the OriginPro 8 software. The differences in IC_{50} values of the parent compounds and metabolites, alone or in mixtures, were assessed using one-way ANOVA. The differences in degradation kinetic parameters and DT_{50} values of parent compounds and metabolites, alone or in mixture, were assessed in the same manner. The normality and homogeneity of variance were checked prior to ANOVA analysis

(Kolmogorov Smirnov's and Levene's tests, respectively, $P < 0.05$). Separate *post hoc* comparisons (Tukey's test, $P < 0.05$) were applied to determine differences in IC_{50} values of the parent compounds and metabolites, alone or in combination, as well as for the kinetic parameters and DT_x of each compound, separately.

3. Results and Discussion

3.1. Biotransformation kinetics of each herbicide by *Bacillus megaterium* Mes11

3.1.1. Nicosulfuron

Preliminary tests showed that Mes11 strain requires glucose under our conditions (resting cells) to completely biotransform nicosulfuron within 20 days in GSM (Figure 1A), indicating that nicosulfuron is not used as a carbon source. It is co-metabolically transformed, as described for all the other nicosulfuron-transforming strains isolated to date [9,22–24,26,33]. A two-step dissipation of nicosulfuron was observed (Figure 1A). The first step corresponded to about 80% of nicosulfuron dissipation within 19 h of incubation, mainly ascribed to adsorption/absorption of nicosulfuron on bacterial biomass (Table S3). The second step (from 20 h to 20 days) corresponded mainly to the quantitative transformation of nicosulfuron into three major metabolites (Figures 1B-D and 2A) identified by LC-MS and 1H NMR analyses. Two of them, **ASDM** and **ADMP**, resulted from cleavage of the sulfonylurea bridge C-N bond. A third metabolite (**N3**), resulting from cleavage of the C-S bond of the sulfonylurea bridge and contraction following elimination of the sulfur dioxide group, was identified as 2-(1-(4,6-dimethoxy-pyrimidin-2-yl)-ureido)-N,N-dimethyl-nicotinamide, as already described [9,23,24]. A very minor metabolite (**N4**, 2-(4,6-dimethoxy-pyrimidin-2-yl)-N,N-dimethyl-nicotinamide) was also detected by LC-(+)ESI-MS, corresponding to the hydrolysis of the **N3** urea function (Figure 2A). **N3** has been already identified with other strains [9,23,24], as well as **N4** [9].

Both ASDM and ADMP reached the highest concentrations (about 0.9 mM after only 8 days of incubation; Figures 1B-C), indicating that this transformation pathway dominates as commonly described [9,34–38]. ADMP, also identified with all the nicosulfuron-transforming strains reported in the literature, can be therefore described as a common metabolite.

3.1.2. Mesotrione

Both HPLC and ^1H NMR analyses revealed complete removal of mesotrione (1 mM) within 5 h of incubation (Figure 3A). The dissipation of mesotrione was ascribed to biotransformation processes because its concentration remained stable in the abiotic control and no significant amount of mesotrione was adsorbed on the bacterial biomass (data not shown). In our study, the biotransformation of mesotrione was four to five-times faster than that observed previously in minimal medium [15], probably due to the presence of glucose (5 g/L), that could lead to a different physiological state of the Mes11 strain. The metabolites of mesotrione identified by ^1H NMR and LC-MS were the same as those previously described [15] (Figure 2B): two transitory metabolites (a hydroxylamino derivative (**M2**) obtained by reduction of the nitro group of mesotrione in equilibrium with its cyclized form isoxazolol (**M3**) due to an intramolecular cyclization of the hydroxyl moiety of the hydroxylamine on the nearest carbonyl group), subsequently being transformed into **AMBA**, which accumulated in the medium (Figure 3B). Therefore, the biotransformation pathway of mesotrione by the Mes11 strain was unchanged by glucose.

The M2 and M3 transitory metabolites reached their maximum concentrations (M2 0.5, M3 0.65 mM) after 8 and 19 h of incubation, respectively, and completely disappeared after 8 and 20 days of incubation (Figures 3C-D). The concentration of AMBA was higher than 0.2 mM after 19 h of incubation, which was about twice the concentration found without glucose (*i.e.* compared to Batisson et al. [15]), which is consistent with the faster transformation of

mesotrione observed in our study. The presence of glucose has therefore not only accelerated the transformation of mesotrione, but also increased the final concentration of its end-product AMBA.

3.1.3. *S-metolachlor*

S-metolachlor was not transformed by the strain Mes11 since its initial and final (after 20 days of incubation) concentrations were similar.

3.2. Biotransformation kinetics of the herbicide mixtures by *B. megaterium* Mes11

The same mesotrione and nicosulfuron metabolites were identified with or without the other herbicides, indicating no effect of the herbicides studied on the biotransformation pathway of nicosulfuron (Figure 2A) and mesotrione (Figure 2B). However, some differences could be identified in terms of transformation kinetics.

The kinetics of nicosulfuron disappearance was well fitted by the DFOP model ($r^2 > 0.93$), which corresponds to a bi-phasic exponential decrease (Figure 1A; Table 1). This model corresponds to the sum of two first order terms (see Materials and Methods section, Equation 1). The term with the faster rate dominates the early degradation step whereas the term with the slower rate dominates the later part of the degradation curve. The DFOP model (also suitable for mono degradation curves [39]) was therefore applied in our study for the transformation of metabolites.

3.2.1. *Effects of mesotrione and/or S-metolachlor on the biotransformation of nicosulfuron*

The first rate constant k_1 of nicosulfuron in the presence of mesotrione (with or without *S-metolachlor*) was 4 to 13-times less than those of nicosulfuron alone or associated with *S-metolachlor*, showing that nicosulfuron dissipation was considerably slowed down by

mesotrione during the first phase of dissipation (Table 1, Figure 1A, Tukey's test, $P < 0.05$), corresponding mainly to biosorption (Table S3). We assume that mesotrione modified the bacterial cell wall properties, leading to a reduction of nicosulfuron adsorption/absorption into the cell, as described in the case of chlorothalonil and atrazine [40]. Lower degradation efficiency of mixed pesticides (methyl-parathion, lindane and carbofuran) compared to individual pesticides was also observed in a previous study [41]. This resulted in lower DT_{50} values for nicosulfuron without mesotrione (Table 1, Figure S1A, Tukey's test, $P < 0.05$).

Conversely, the second rate constant k_2 of nicosulfuron in the presence of mesotrione (with or without S-metolachlor) was higher than those of nicosulfuron alone or with S-metolachlor during the second phase of dissipation (from 19 h to 20 days of incubation) (Table 1, Figure 1A, Tukey's test, $P < 0.05$). This stimulation indicates a positive effect of mesotrione on the nicosulfuron biotransformation, which is predominant during this phase. Nevertheless, as mesotrione disappeared very quickly (within 5 h), the effect observed is probably due to mesotrione metabolites rather than to the parent compound.

The DT_{90} value of nicosulfuron alone was the lowest one, showing an overall inhibitory effect of the other two herbicides on the biotransformation of nicosulfuron (Table 1, Figure S1B, Tukey's test, $P < 0.05$).

Some differences have also been observed for production kinetics of metabolites in the mixtures, as in the case of ASDM formation, which was accelerated in the presence of S-metolachlor (independently of mesotrione), as illustrated by the p rate constant (Table 1, Figure 1B, Tukey's test, $P < 0.05$). The maximum concentrations of ASDM and ADMP were also lowest in the case of the ternary mixture (Table 1, Figure 1C, Tukey's test, $P < 0.05$). Overall, these results did not show any significant effect of mesotrione and/or S-metolachlor on the balance between the two identified biotransformation pathways of nicosulfuron leading to ADMP/ASDM or N3.

3.2.2. Effects of nicosulfuron and/or S-metolachlor on the biotransformation of mesotrione

The disappearance of mesotrione was not modified in the presence of nicosulfuron and/or S-metolachlor (Figure 3A, Table 2, Tukey's test, $P < 0.05$). However, some significant differences were observed for the kinetic parameters of the formation/disappearance of M2 and M3 (Table 2, Figures 3C-D, Tukey's test, $P < 0.05$) for different conditions. It is difficult to conclude on these differences, because these compounds are transitory metabolites and their kinetic parameters depend on many unknown phenomena, such as dynamic equilibrium between M2 and M3, flux from mesotrione and to AMBA.

Interestingly, the formation of the end-product AMBA was faster in the presence of S-metolachlor than in its absence, as shown by the p rate constant and t_{50} values (Table 2, Figure 3B, Tukey's test, $P < 0.05$). The production of AMBA results from a nitroreduction of the nitro group of mesotrione. S-metolachlor may thus induce the expression of the NfrA1 and NfrA2 nitroreductases [42], since previous studies have shown an activation of the nitroreductase regulatory system by pesticides such as paraquat [43]. However, although it accelerates the transformation of mesotrione into AMBA, S-metolachlor led to a lower final AMBA concentration (C_{max}), mainly with the ternary mixture (Table 2, Figure 3B, Tukey's test, $P < 0.05$). These results showed that S-metolachlor can modify the formation kinetics of AMBA, which is more persistent in soil than mesotrione [6].

The identification and quantification of metabolites are thus of prime concern, mainly if they are toxic compounds causing adverse effects to the environment. Then, new data on the biodegradation and toxicity of pesticide (or other compounds) mixtures could be added in databases hosted by national (e.g. E-Phy of ANSES (Agence Nationale de Sécurité Sanitaire de l'Alimentation, de l'Environnement et du Travail) in France) or European institutions such as EFSA (European Food and Safety Authority). To our knowledge, such databases

regrouping biodegradation and toxicity data of mixtures do not exist so far, and have to be created in order to improve knowledges on the impact of hazardous compound mixtures towards target and non-target organisms in the environment.

3.3. Toxicity of herbicide and/or metabolite mixtures

The experimental data of mixture toxicity were compared with those predicted by the CA and IA models. CA model is known to generate larger predicted mixture effects than IA, and is therefore considered as more conservative [44–46] and adapted to multicomponent (> two molecules) mixtures [47,48]. However, the combined variances of toxicity data for individual compounds and mixtures render detection of small deviations from the reference models (CA and IA) difficult. Biologically significant synergy was therefore previously defined as a more than two fold deviation from CA or IA [44,45,49] and evaluated with the MDR values chosen as the reference scale because of the simplicity of adjusting values from the predictive model. Risk assessors can easily divide the level of potential toxicity described by a mixture model by the MDR to adjust empirical data for the mixture of interest [44].

3.3.1. Toxicity of the herbicide mixtures (starting conditions)

The MDR values of all the parent herbicide mixtures tested were ≥ 2 whatever the model tested (CA and IA). These results suggest synergistic effects of these compounds for the toxicity towards *A. fischeri* (Table 3). Moreover, IC_{50} values of the single parent compounds were about 3-fold (mesotrione, Figure S2A), 4 to 10-fold (nicosulfuron, Figure S2B) and 6 to 15-fold (S-metolachlor, Figure S2C) higher than those of their binary or ternary mixtures (Tukey's test, $P < 0.05$). This was already emphasized by Cedergreen [45] who showed five groups of pesticides over-represented in the synergistic mixtures (azole fungicides, triazine herbicides and organophosphate, carbamate and pyrethroid insecticides). The results of the

present study indicated that β -triketone, sulfonylurea and chloroacetanilide herbicides, which have different modes of action in plants, are potential synergistic compounds at high concentration on the bioluminescent marine bacteria *A. fischeri*, a model strain in microbial ecotoxicology. However, the mode of action of these herbicides in microorganisms is still unknown. The IC_{50} found in our study were relatively high (of μM order) compared to the environmental concentrations of mesotrione, nicosulfuron and S-metolachlor detected in aquatic environments (of nM order, [13,14]). Nevertheless, these concentrations are of the order of those potentially found in soil during or just after the herbicide treatment, indicating a potential risk for edaphic microorganisms. These results may also permit the mechanism of action of these compounds in microorganisms to be identified in the future. This has been very poorly documented so far. Conversely, in higher organisms, several mechanisms have been proposed (change of bioavailability, uptake, internal transportation, metabolism, binding at the target site and excretion of each compound) [45].

3.3.2. Toxicity of metabolite and/or S-metolachlor mixtures (final conditions)

The toxicity of metabolite mixtures was determined with and without S-metolachlor because this herbicide was not degraded by the strain Mes11 and was therefore present at the end of the experiment. According to CA prediction, synergistic effects were observed in the case of a mixture composed of AMBA + ASDM (independently of the presence of S-metolachlor), as shown by $MDR \geq 2$ (Table 3). On the contrary, the effect of the other mixtures was mainly antagonistic or additive ($MDR < 0.5$ or 1), depending on each mixture (Table 3). Overall, the MDR values calculated with IA model were similar or lower than those obtained with CA. Consequently, synergism and additivity were most often predicted by CA than IA, whereas antagonism was found for a higher number of metabolite mixtures with IA model compared to CA (Figure S3). These results are in accordance with other studies

describing CA as more conservative than IA [44–46]. Moreover, the IA model is based on the Bliss Independence approach which presents some limitations. First, the determination of mixture effects implies the knowledge of the action mechanism of each component in the mixture, which is not the case for the majority of compounds that can possess complex, multiple and possibly unknown action mechanisms. Then, Bliss Independence approach assumes that dose response curves are exponential, which is not necessary the case. In the present study, dose response curves were fitted with a sigmoid function, allowing the determination of IC_{50} values. The dose response curves obtained were parallel on a log-dose scale (i.e. constant ratio of dose at each level of effect) and have equal maximum effect for an individual compound, which conditions the application of Loewe Additivity (CA model). Therefore, CA model seems to be more suitable for the prediction of antagonism/additivity/synergism in the case of pesticide/metabolite mixtures in the present study.

The IC_{50} values of a given compound in each mixture were also compared. There was no effect of the other compounds on the IC_{50} value of AMBA, except for two mixtures (AMBA + S-metolachlor + ADMP or ASDM) being about twice as toxic as the single compound (Figure S2D, Tukey's test, $P < 0.05$). The IC_{50} values of ADMP in all the tested mixtures were about 6.5-fold (ADMP + AMBA + S-metolachlor) to 14-fold (ADMP + ASDM) higher than that of the single compound (Figure S2E, Tukey's test, $P < 0.05$). On the contrary, the IC_{50} value of ASDM ($> 3900 \mu\text{M}$) indicates extremely low toxicity towards *A. fischeri* that was considerably increased in all mixtures tested (up to 100 to 200 μM), and to a lesser extent in the mixture composed of ASDM + ADMP (about 400 μM) (Figure S2F, Tukey's test, $P < 0.05$).

To date, studies related to the toxicity of pesticide metabolites towards *A. fischeri* were only performed with individual compounds (e.g. [7,50,51]). The present work showed

synergism and additivity for AMBA and ASDM mixtures, and antagonism for the majority of the other metabolite mixtures tested. For more complete toxicity analyses, it would be interesting in the future to include the other metabolites, although they are present in very low concentrations and/or as intermediates.

3.4. Conclusions

The present work characterizes mixture effects on the biotransformation and toxicity of three herbicides (mesotrione, nicosulfuron, S-metolachlor) applied on maize crops. Our results indicate that *B. megaterium* Mes11 was able to simultaneously biotransform two herbicides belonging to different chemical families (β -triketone and sulfonyleurea), which is poorly described so far. Unless the biotransformation pathways of each herbicide (*i.e.* the identified metabolites) were not modified in the presence of the other herbicides studied, differences in active ingredient biotransformation and metabolite production kinetics were observed. The toxicity tests also revealed mixture effects, leading to synergistic interactions for parent compounds and synergistic/additive/antagonistic effects for metabolites.

The present study gives new data on the toxicity of mesotrione, nicosulfuron (and their metabolites) and S-metolachlor, alone or in binary/ternary mixtures towards *A. fischeri*, allowing a better understanding of their potential impact on microorganisms. New toxicity data on the herbicides studied and their metabolites can also be used to identify synergistic compounds, and thus should perhaps be considered even if active ingredients occur at concentrations below toxic level in the environment. In a general way, our study emphasizes the importance to take into account the 'pesticidome' (mixture including the active ingredients, metabolites and adjuvants potentially co-occurring in the environment) and not only the active ingredient, to assess the overall fate and toxicity of pesticides in the

environment. New data could then be used to improve the global risk assessment of pesticide mixtures.

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Conflict of Interest

The authors declare no competing financial interest.

References

- [1] A. Kienzler, E. Berggren, J. Bessems, S. Bopp, S. van der Linden, A. Worth, Assessment of mixtures-Review of regulatory requirements and guidance, JRC Rep. ISSN 1831-9424. (2014).
http://green.pidc.org.tw/upload/news/EU_Assessment%20of%20Mixtures%20-%20Review%20of%20Regulatory%20Requirements%20and%20Guidance.pdf (accessed October 6, 2016).
- [2] A. Kienzler, S. Bopp, S. van der Linden, E. Berggren, A. Worth, Regulatory assessment of chemical mixtures: Requirements, current approaches and future perspectives, Regul. Toxicol. Pharmacol. 80 (2016) 321–334.
- [3] G. Mitchell, D.W. Bartlett, T.E. Fraser, T.R. Hawkes, D.C. Holt, J.K. Townson, R.A. Wichert, Mesotrione: a new selective herbicide for use in maize, Pest Manag. Sci. 57 (2001) 120–128.
- [4] P. Alferness, L. Wiebe, Determination of mesotrione residues and metabolites in crops, soil, and water by liquid chromatography with fluorescence detection, J. Agric. Food Chem. 50 (2002) 3926–3934.
- [5] E. Dumas, M. Giraud, E. Goujon, M. Halma, E. Khili, M. Stauffert, I. Batisson, P. Besse-Hoggan, J. Bohatier, P. Bouchard, H. Celle-Jeanton, M. Costa Gomes, F. Delbac, C. Forano, P. Goupil, N. Guix, P. Husson, G. Ledoigt, C. Mallet, C. Mousty, V. Prévot, C. Richard, S. Sarraute, Fate and ecotoxicological impact of new generation herbicides from the triketone family: An overview to assess the environmental risks, J. Hazard. Mater. 325 (2017) 136–156.
- [6] H. Barchanska, A. Kluza, K. Krajczewska, J. Maj, Degradation study of mesotrione and other triketone herbicides on soils and sediments, J. Soils Sediments. 16 (2016) 125–133.
- [7] J.L. Bonnet, F. Bonnemoy, M. Dusser, J. Bohatier, Toxicity assessment of the herbicides sulcotrione and mesotrione toward two reference environmental microorganisms: *Tetrahymena pyriformis* and *Vibrio fischeri*, Arch. Environ. Contam. Toxicol. 55 (2008) 576–583.
- [8] J.V. Schloss, Acetolactate synthase, mechanism of action and its herbicide binding site, Pestic. Sci. 29 (1990) 283–292.
- [9] L. Carles, M. Joly, F. Bonnemoy, M. Lereboure, I. Batisson, P. Besse-Hoggan, Identification of sulfonylurea biodegradation pathways enabled by a novel nicosulfuron-transforming strain *Pseudomonas fluorescens* SG-1: Toxicity assessment and effect of formulation, J. Hazard. Mater. 324 (2017) 184–193. doi:10.1016/j.jhazmat.2016.10.048.
- [10] T. Gotz, P. Boger, The very-long-chain fatty acid synthase is inhibited by chloroacetamides, Z. Naturforschung C. 59 (2004) 549–553.
- [11] N. Baran, L. Gourcy, Sorption and mineralization of S-metolachlor and its ionic metabolites in soils and vadose zone solids: Consequences on groundwater quality in an alluvial aquifer (Ain Plain, France), J. Contam. Hydrol. 154 (2013) 20–28.
- [12] D.K. Kolpin, D.J. Schnoebelen, E.M. Thurman, Degradates provide insight to spatial and temporal trends of herbicides in ground water, Ground Water. 42 (2004) 601–608.
- [13] ADES, Portail national d'accès aux données sur les eaux souterraines.
<http://www.ades.eaufrance.fr/ExportData.aspx?paramFrance=ok.> (2017).
<http://www.ades.eaufrance.fr/ExportData.aspx?paramFrance=ok> (accessed October 5, 2017).

- [14] NAIADES, Données sur la qualité des eaux de surface.
<http://www.naiades.eaufrance.fr/acces-donnees#/physicochimie.>, (2017).
<http://www.naiades.eaufrance.fr/acces-donnees#/physicochimie> (accessed October 5, 2017).
- [15] I. Batisson, O. Crouzet, P. Besse-Hoggan, M. Sancelme, J.-F. Mangot, C. Mallet, J. Bohatier, Isolation and characterization of mesotrione-degrading *Bacillus* sp. from soil, *Environ. Pollut.* 157 (2009) 1195–1201.
- [16] S. Durand, M. Sancelme, P. Besse-Hoggan, B. Combourieu, Biodegradation pathway of mesotrione: Complementarities of NMR, LC–NMR and LC–MS for qualitative and quantitative metabolic profiling, *Chemosphere.* 81 (2010) 372–380.
- [17] S. Durand, P. Amato, M. Sancelme, A.-M. Delort, B. Combourieu, P. Besse-Hoggan, First isolation and characterization of a bacterial strain that biotransforms the herbicide mesotrione, *Lett. Appl. Microbiol.* 43 (2006) 222–228.
- [18] J. Liu, S. Chen, J. Ding, Y. Xiao, H. Han, G. Zhong, Sugarcane bagasse as support for immobilization of *Bacillus pumilus* HZ-2 and its use in bioremediation of mesotrione-contaminated soils, *Appl. Microbiol. Biotechnol.* 99 (2015) 10839–10851.
- [19] M. Pileggi, S.A. Veiga Pileggi, L.R. Olchanheski, P.A. Garbugio da Silva, A.M.M. Gonzalez, W.C. Koskinen, B. Barber, M.J. Sadowsky, Isolation of mesotrione-degrading bacteria from aquatic environments in Brazil, *Chemosphere.* 86 (2012) 1127–1132.
doi:10.1016/j.chemosphere.2011.12.041.
- [20] S. Romdhane, M. Devers-Lamrani, F. Martin-Laurent, C. Calvayrac, E. Rocaboy-Faquet, D. Riboul, J.-F. Cooper, L. Barthelmebs, Isolation and characterization of *Bradyrhizobium* sp. SR1 degrading two β -triketone herbicides, *Environ. Sci. Pollut. Res.* 23 (2015) 4138–4148.
- [21] Y. Yang, B. Tao, W. Zhang, J. Zhang, Isolation and screening of microorganisms capable of degrading nicosulfuron in water, *Front. Agric. China.* 2 (2008) 224–228.
doi:10.1007/s11703-008-0033-3.
- [22] H. Zhang, W. Mu, Z. Hou, X. Wu, W. Zhao, X. Zhang, H. Pan, S. Zhang, Biodegradation of nicosulfuron by the bacterium *Serratia marcescens* N80, *J. Environ. Sci. Health Part B.* 47 (2012) 153–160.
- [23] J. Song, J. Gu, Y. Zhai, W. Wu, H. Wang, Z. Ruan, Y. Shi, Y. Yan, Biodegradation of nicosulfuron by a *Talaromyces flavus* LZM1, *Bioresour. Technol.* 140 (2013) 243–248.
- [24] W. Zhao, C. Wang, L. Xu, C. Zhao, H. Liang, L. Qiu, Biodegradation of nicosulfuron by a novel *Alcaligenes faecalis* strain ZWS11, *J. Environ. Sci.* 35 (2015) 151–162.
- [25] W. Zhao, L. Xu, D. Li, X. Li, C. Wang, M. Zheng, C. Pan, L. Qiu, Biodegradation of thifensulfuron-methyl by *Ochrobactrum* sp. in liquid medium and soil, *Biotechnol. Lett.* 37 (2015) 1385–1392.
- [26] L. Wang, X. Zhang, Y. Li, Degradation of nicosulfuron by a novel isolated bacterial strain *Klebsiella* sp. Y1: condition optimization, kinetics and degradation pathway, *Water Sci. Technol.* 73 (2016) 2896–2903.
- [27] P.F. Martins, C.O. Martinez, G. de Carvalho, P.I.B. Carneiro, R.A. Azevedo, S.A.V. Pileggi, I.S. de Melo, M. Pileggi, Selection of microorganisms degrading S-Metolachlor herbicide, *Braz. Arch. Biol. Technol.* 50 (2007) 153–159.
- [28] P. Joly, F. Bonnemoy, J.-C. Charvy, J. Bohatier, C. Mallet, Toxicity assessment of the maize herbicides S-metolachlor, benoxacor, mesotrione and nicosulfuron, and their corresponding commercial formulations, alone and in mixtures, using the Microtox® test, *Chemosphere.* 93(10) (2013) 2444–2450.

- [29] D. Spatz, B.C. ERB, K. White, D. Young, Standard Operating Procedure for Using the NAFTA Guidance to Calculate Representative Half-life Values and Characterizing Pesticide Degradation, (2015).
- [30] AZUR-Environmental, Microtox user's manual AZUR Environmental, Carlsbad, CA, USA, (1996).
- [31] K. Doe, P. Jackman, R. Scroggins, D. McLeay, G. Wohlgeschaffen, Solid-phase test for sediment toxicity using the luminescent bacterium, *Vibrio Fischeri*, in: C. Blaise, J.-F. Férard (Eds.), Small-Scale Freshw. Toxic. Investig., Springer-Verlag, Berlin/Heidelberg, 2005: pp. 107–136.
- [32] M.C. Berenbaum, The expected effect of a combination of agents: the general solution, *J. Theor. Biol.* 114 (1985) 413–431.
- [33] X.H. Lu, Z.H. Kang, B. Tao, Y.N. Wang, J.G. Dong, J.L. Zhang, Degradation of nicosulfuron by *Bacillus subtilis* YB1 and *Aspergillus niger* YF1, *Appl. Biochem. Microbiol.* 48 (2012) 460–466.
- [34] A.K. Sarmah, J. Sabadie, Hydrolysis of sulfonylurea herbicides in soils and aqueous solutions: a review, *J. Agric. Food Chem.* 50 (2002) 6253–6265.
- [35] J. Kim, K.-H. Liu, S.-H. Kang, S.-J. Koo, J.-H. Kim, Degradation of the sulfonylurea herbicide LGC-42153 in flooded soil, *Pest Manag. Sci.* 59 (2003) 1037–1042.
- [36] W. Luo, Y. Zhao, H. Ding, X. Lin, H. Zheng, Co-metabolic degradation of bensulfuron-methyl in laboratory conditions, *J. Hazard. Mater.* 158 (2008) 208–214.
- [37] J.-P. Ma, Z. Wang, P. Lu, H. Wang, S. Waseem Ali, S.-P. Li, X. Huang, Biodegradation of the sulfonylurea herbicide chlorimuron-ethyl by the strain *Pseudomonas* sp. LW3, *FEMS Microbiol. Lett.* 296 (2009) 203–209.
- [38] J. Xu, X. Li, Y. Xu, L. Qiu, C. Pan, Biodegradation of pyrazosulfuron-ethyl by three strains of bacteria isolated from contaminated soils, *Chemosphere.* 74 (2009) 682–687.
- [39] EPA, Standard operating procedure for using the NAFTA guidance to calculate representative half-life values and characterizing pesticide degradation. <https://www.epa.gov/pesticide-science-and-assessing-pesticide-risks/standard-operating-procedure-using-nafta-guidance>, US EPA. (2015).
- [40] M.E. DeLorenzo, L. Serrano, Individual and mixture toxicity of three pesticides; atrazine, chlorpyrifos, and chlorothalonil to the marine phytoplankton species *Dunaliella tertiolecta*, *J. Environ. Sci. Health Part B.* 38 (2003) 529–538.
- [41] K. Rama Krishna, L. Philip, Bioremediation of single and mixture of pesticide-contaminated soils by mixed pesticide-enriched cultures, *Appl. Biochem. Biotechnol.* 164 (2011) 1257–1277.
- [42] L. Carles, P. Besse-Hoggan, M. Joly, A. Vigouroux, S. Morera, I. Batisson, Functional and structural characterization of two *Bacillus megaterium* nitroreductases biotransforming the herbicide mesotrione, *Biochem. J.* 473 (2016) 1443–1453.
- [43] M.D. Roldán, E. Pérez-Reinado, F. Castillo, C. Moreno-Vivián, Reduction of polynitroaromatic compounds: the bacterial nitroreductases, *FEMS Microbiol. Rev.* 32 (2008) 474–500.
- [44] J.B. Belden, R.J. Gilliom, M.J. Lydy, How well can we predict the toxicity of pesticide mixtures to aquatic life?, *Integr. Environ. Assess. Manag.* 3 (2007) 364–372.
- [45] N. Cedergreen, Quantifying synergy: a systematic review of mixture toxicity studies within environmental toxicology, *Plos One.* 9 (2014) e96580.
- [46] N. Cedergreen, A.M. Christensen, A. Kamper, P. Kudsk, S.K. Mathiassen, J.C. Streibig, H. Sørensen, A review of independent action compared to concentration addition as reference models for mixtures of compounds with different molecular target sites, *Environ. Toxicol. Chem.* 27 (2008) 1621–1632.

- [47] J.W. Deneer, Toxicity of mixtures of pesticides in aquatic systems, *Pest Manag. Sci.* 56 (2000) 516–520.
- [48] M.S.J. Warne, D.W. Hawker, The number of components in a mixture determines whether synergistic and antagonistic or additive toxicity predominate: The funnel hypothesis, *Ecotoxicol. Environ. Saf.* 31 (1995) 23–28.
- [49] M. Wiczerzak, B. Kudlak, G. Yotova, M. Nedyalkova, S. Tsakovski, V. Simeonov, J. Namieśnik, Modeling of pharmaceuticals mixtures toxicity with deviation ratio and best-fit functions models, *Sci. Total Environ.* 571 (2016) 259–268.
- [50] C. Tixier, M. Sancelme, M. Sancelme, F. Bonnemoy, A. Cuer, H. Veschambre, Degradation products of a phenylurea herbicide, diuron: Synthesis, ecotoxicity, and biotransformation, *Environ. Toxicol. Chem.* 20 (2001) 1381–1389.
- [51] O. Osano, W. Admiraal, H.J.C. Klamer, D. Pastor, E.A.J. Bleeker, Comparative toxic and genotoxic effects of chloroacetanilides, formamidines and their degradation products on *Vibrio fischeri* and *Chironomus riparius*, *Environ. Pollut.* 119 (2002) 195–202.

Figure captions

Figure 1. Comparative biotransformation kinetics of nicosulfuron (alone or in mixtures) by *Bacillus megaterium* Mes11. Nicosulfuron alone (—■—), with mesotrione (—●—), S-metolachlor (—▲—) or mesotrione and S-metolachlor (—▼—). (A) nicosulfuron, (B) ASDM, 2-(aminosulfonyl)-N,N-dimethyl-3-pyridinecarboxamide (C) ADMP, 2-amino-4,6-dimethoxypyrimidine and (D) N3, 2-(1-(4,6-dimethoxy-pyrimidin-2-yl)-ureido)-N,N-dimethyl-nicotinamide. The values are means \pm standard errors ($n \geq 3$) of experimental data. Fitting curves are also indicated (decreasing concentration = DFOP model, increasing concentration = DoseResp model)..

Figure 2. Proposed scheme of (A) nicosulfuron and (B) mesotrione biotransformation by *Bacillus megaterium* Mes11 in GSM. The thick and thin arrows show the main and the minor pathways, respectively. ADMP, 2-amino-4,6-dimethoxypyrimidine; ASDM, 2-(aminosulfonyl)-N,N-dimethyl-3-pyridinecarboxamide; N3, 2-(1-(4,6-dimethoxy-pyrimidin-2-yl)-ureido)-N,N-dimethyl-nicotinamide; N4, 2-(4,6-dimethoxy-pyrimidin-2-yl)-N,N-dimethyl-nicotinamide; AMBA, 2-Amino-4-(methylsulfonyl)benzoic acid; M2 and M3, hydroxylamino and isoxazolol derivatives of mesotrione, respectively.

Figure 3. Comparative biotransformation kinetics of mesotrione (alone or in mixtures) by *Bacillus megaterium* Mes11. Mesotrione alone (—■—), with nicosulfuron (—●—), S-metolachlor (—▲—) or nicosulfuron and S-metolachlor (—▼—). (A) mesotrione, (B) AMBA, 2-Amino-4-(methylsulfonyl)benzoic acid (C) M2 and (D) M3, hydroxylamino and isoxazolol derivatives of mesotrione, respectively. The values are means \pm standard errors (n

≥ 3) of experimental data. Fitting curves are also indicated (decreasing concentration = DFOP model, increasing concentration = DoseResp model)

ACCEPTED MANUSCRIPT

Figure 1

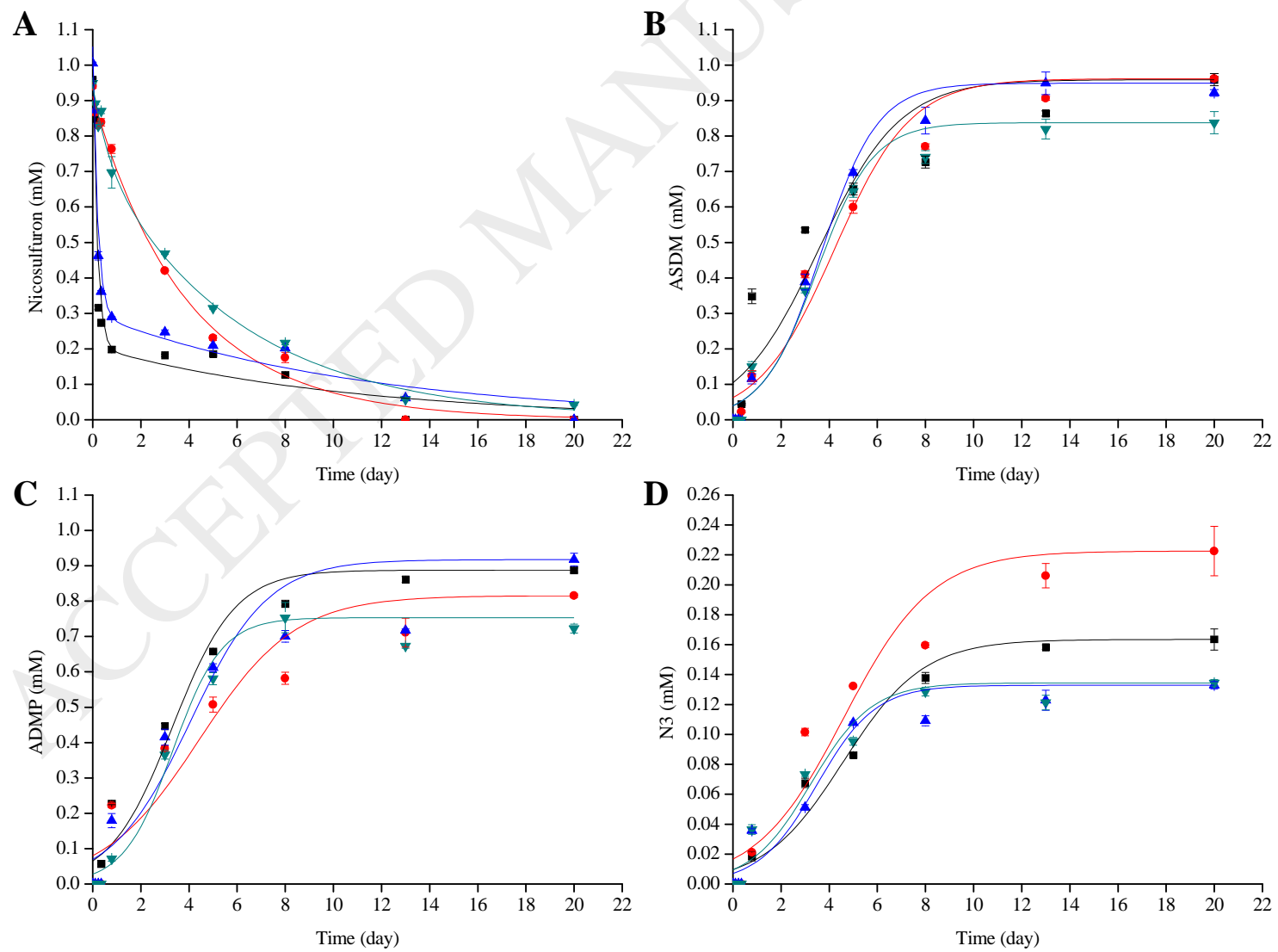


Figure 2

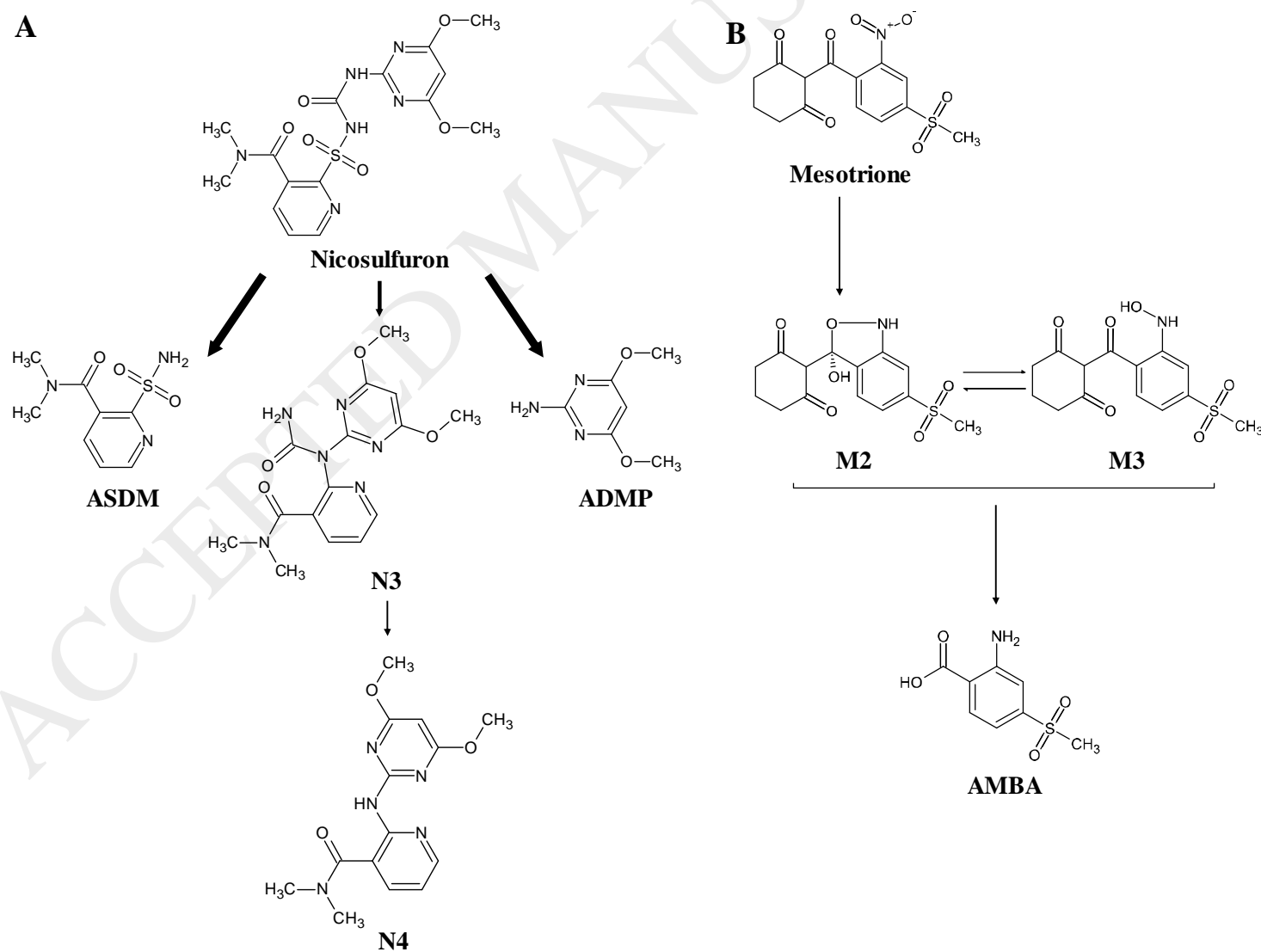
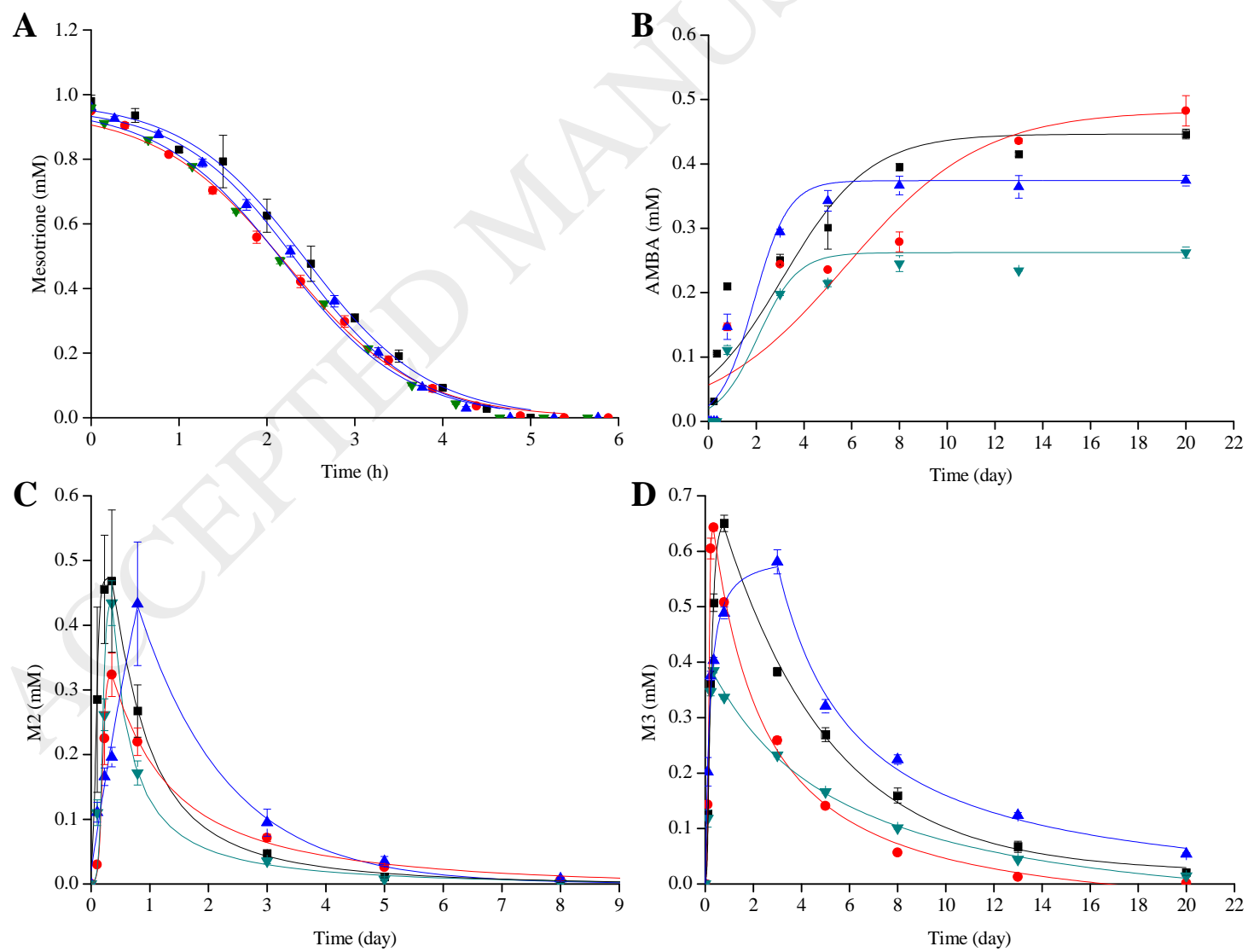


Figure 3



Tables

Table 1. Kinetics of nicosulfuron biotransformation and production of its metabolites. The experimental data were fitted with the DFOP and DoseResp function using CAKE and OriginPro, respectively, as described in the Materials and Methods section. The values of model parameters, coefficient of determination (r^2) and Dissipation Time x% (DT_x) are reported as the mean \pm standard error (SE), $n = 3$. Significant differences between conditions for the parameters of each compound are indicated by lowercase letters $a < b < c < d$ (Tukey's test, $P < 0.05$).

Compound	Condition	Model	Model parameters				DT_{50} (day)	DT_{90} (day)	r^2
			C_0 (mM)	k_1 (day^{-1})	k_2 (day^{-1})	g			
Nicosulfuron	Nicosulfuron	DFOP	$1.02 \pm 4 \cdot 10^{-3}$	5.35 ± 0.02 (b)	$0.09 \pm 1 \cdot 10^{-3}$ (a)	0.80 ± 0.01	$0.18 \pm 3 \cdot 10^{-3}$ (a)	7.41 ± 0.32 (a)	0.937 ± 0.002
	Nicosulfuron + Mesotrione	DFOP	0.91 ± 0.01	0.41 ± 0.11 (a)	$0.23 \pm 3 \cdot 10^{-3}$ (c)	0.30 ± 0.22	2.72 ± 0.08 (b)	9.26 ± 0.34 (b)	0.994 ± 0.002
	Nicosulfuron + S-metolachlor	DFOP	$1.05 \pm 2 \cdot 10^{-3}$	5.02 ± 0.10 (b)	$0.09 \pm 2 \cdot 10^{-3}$ (a)	$0.72 \pm 1 \cdot 10^{-3}$	$0.23 \pm 4 \cdot 10^{-3}$ (a)	11.73 ± 0.23 (c)	0.965 ± 0.002
	Nicosulfuron + Mesotrione + S-metolachlor	DFOP	0.94 ± 0.01	1.38 ± 0.41 (a)	$0.16 \pm 4 \cdot 10^{-3}$ (b)	0.23 ± 0.02	2.83 ± 0.18 (b)	12.47 ± 0.15 (c)	0.993 ± 0.003
			Model parameters						
			C_{max} (mM)	p (day^{-1})	h_{50} (day)				
ASDM	Nicosulfuron	DoseResp	0.96 ± 0.02 (b)	0.26 ± 0.03 (a)	3.59 ± 0.25 (a)	-	-	-	0.852 ± 0.017
	Nicosulfuron + Mesotrione	DoseResp	0.96 ± 0.01 (b)	$0.27 \pm 2 \cdot 10^{-4}$ (a)	4.22 ± 0.01 (a)	-	-	-	0.966 ± 0.001
	Nicosulfuron + S-metolachlor	DoseResp	0.96 ± 0.02 (b)	$0.37 \pm 8 \cdot 10^{-4}$ (b)	3.71 ± 0.12 (a)	-	-	-	0.982 ± 0.003
	Nicosulfuron + Mesotrione + S-metolachlor	DoseResp	0.84 ± 0.03 (a)	0.37 ± 0.01 (b)	3.50 ± 0.12 (a)	-	-	-	0.978 ± 0.002
ADMP	Nicosulfuron	DoseResp	0.89 ± 0.01 (b)	$0.33 \pm 3 \cdot 10^{-3}$ (ab)	3.31 ± 0.03 (a)	-	-	-	0.966 ± 0.001
	Nicosulfuron + Mesotrione	DoseResp	0.82 ± 0.01 (ab)	0.21 ± 0.02 (a)	4.44 ± 0.22 (b)	-	-	-	0.901 ± 0.019
	Nicosulfuron + S-metolachlor	DoseResp	0.92 ± 0.02 (b)	0.27 ± 0.01 (a)	4.04 ± 0.08 (b)	-	-	-	0.904 ± 0.014
	Nicosulfuron + Mesotrione + S-metolachlor	DoseResp	0.76 ± 0.03 (a)	0.43 ± 0.04 (b)	3.36 ± 0.21 (a)	-	-	-	0.975 ± 0.008
N3	Nicosulfuron	DoseResp	0.17 ± 0.01 (b)	0.26 ± 0.01 (a)	4.69 ± 0.09 (b)	-	-	-	0.969 ± 0.004
	Nicosulfuron + Mesotrione	DoseResp	0.22 ± 0.02 (c)	0.24 ± 0.04 (a)	4.61 ± 0.54 (b)	-	-	-	0.941 ± 0.009
	Nicosulfuron + S-metolachlor	DoseResp	$0.13 \pm 3 \cdot 10^{-3}$ (a)	0.36 ± 0.01 (b)	3.51 ± 0.11 (a)	-	-	-	0.943 ± 0.011
	Nicosulfuron + Mesotrione + S-metolachlor	DoseResp	$0.14 \pm 2 \cdot 10^{-3}$ (a)	$0.34 \pm 3 \cdot 10^{-3}$ (b)	3.26 ± 0.03 (a)	-	-	-	0.946 ± 0.003

k_1 , k_2 and p : rate constants

Table 2. Kinetics of mesotrione biotransformation and production of its metabolites. The experimental data were fitted with the DFOP and DoseResp function using CAKE and OriginPro, respectively, as described in the Materials and Methods section. The values of model parameters, coefficient of determination (r^2) and Dissipation Time x% (DT_x) are reported as the mean \pm standard error (SE), $n = 3$. Significant differences between conditions for the parameters of each compound are indicated by lowercase letters $a < b < c$ (Tukey's test, $P < 0.05$).

Compound	Condition	Model	Model parameters				DT_{50} (day)	DT_{90} (day)	r^2
			C_0 (mM)	k_1 (day^{-1})	k_2 (day^{-1})	g			
M2 ⁽¹⁾	Mesotrione	DFOP	0.51 ± 0.10	0.78 ± 0.07 (b)	$0.01 \pm 8 \cdot 10^{-4}$ (a)	1.00 ± 0.00	0.91 ± 0.09 (a)	3.00 ± 0.30 (a)	0.955 ± 0.021
	Mesotrione + Nicosulfuron	DFOP	0.33 ± 0.03	0.51 ± 0.01 (a)	$0.01 \pm 3 \cdot 10^{-4}$ (a)	1.00 ± 0.00	1.36 ± 0.02 (b)	4.51 ± 0.07 (b)	0.931 ± 0.016
	Mesotrione + S-metolachlor	DFOP	0.43 ± 0.10	0.51 ± 0.00 (a)	$0.01 \pm 5 \cdot 10^{-4}$ (a)	1.00 ± 0.00	1.36 ± 0.00 (b)	4.53 ± 0.00 (b)	0.900 ± 0.000
	Mesotrione + Nicosulfuron + S-metolachlor	DFOP	0.61 ± 0.16	0.85 ± 0.01 (b)	$0.01 \pm 9 \cdot 10^{-4}$ (a)	1.00 ± 0.00	0.82 ± 0.01 (a)	2.72 ± 0.04 (a)	0.928 ± 0.004
M3 ⁽¹⁾	Mesotrione	DFOP	0.65 ± 0.01	0.18 ± 0.01 (b)	0.06 ± 0.06 (a)	$0.99 \pm 7 \cdot 10^{-3}$	3.97 ± 0.29 (b)	13.17 ± 0.96 (b)	0.966 ± 0.009
	Mesotrione + Nicosulfuron	DFOP	0.64 ± 0.01	0.31 ± 0.01 (c)	0.11 ± 0.10 (a)	0.68 ± 0.32	2.27 ± 0.05 (a)	7.54 ± 0.15 (a)	0.979 ± 0.005
	Mesotrione + S-metolachlor	DFOP	0.58 ± 0.02	$0.12 \pm 4 \cdot 10^{-4}$ (a)	$0.11 \pm 4 \cdot 10^{-4}$ (a)	$0.73 \pm 1 \cdot 10^{-3}$	5.84 ± 0.02 (c)	19.40 ± 0.06 (c)	0.931 ± 0.001
	Mesotrione + Nicosulfuron + S-metolachlor	DFOP	$0.38 \pm 4 \cdot 10^{-3}$	$0.17 \pm 2 \cdot 10^{-3}$ (b)	$3 \cdot 10^{-11} \pm 2 \cdot 10^{-11}$ (a)	$0.99 \pm 3 \cdot 10^{-3}$	4.14 ± 0.06 (b)	13.90 ± 0.26 (b)	0.976 ± 0.002
			Model parameters						
			C_{max} (mM)	p^*	h_{50} (day)				
Mesotrione	Mesotrione	DoseResp	0.98 ± 0.02	-0.65 ± 0.06 (a)	2.43 ± 0.13 (a)	-	-	0.989 ± 0.005	
	Mesotrione + Nicosulfuron	DoseResp	0.95 ± 0.01	-0.59 ± 0.02 (a)	2.22 ± 0.05 (a)	-	-	0.995 ± 0.001	
	Mesotrione + S-metolachlor	DoseResp	0.96 ± 0.01	-0.65 ± 0.01 (a)	2.35 ± 0.06 (a)	-	-	0.997 ± 0.001	
	Mesotrione + Nicosulfuron + S-metolachlor	DoseResp	0.96 ± 0.01	$-0.62 \pm 5 \cdot 10^{-4}$ (a)	2.20 ± 0.02 (a)	-	-	0.996 ± 0.001	
AMBA	Mesotrione	DoseResp	0.45 ± 0.01 (c)	0.25 ± 0.04 (a)	3.17 ± 0.37 (b)	-	-	0.880 ± 0.01	
	Mesotrione + Nicosulfuron	DoseResp	0.48 ± 0.02 (c)	0.15 ± 0.01 (a)	5.74 ± 0.28 (c)	-	-	0.865 ± 0.01	
	Mesotrione + S-metolachlor	DoseResp	0.37 ± 0.01 (b)	0.61 ± 0.05 (b)	1.92 ± 0.06 (a)	-	-	0.922 ± 0.02	
	Mesotrione + Nicosulfuron + S-metolachlor	DoseResp	0.26 ± 0.01 (a)	0.53 ± 0.06 (b)	2.09 ± 0.17 (ab)	-	-	0.912 ± 0.02	
M2 ⁽²⁾	Mesotrione	DoseResp	0.51 ± 0.10 (a)	57.49 ± 20.46 (b)	0.13 ± 0.04 (a)	-	-	0.979 ± 0.02	
	Mesotrione + Nicosulfuron	DoseResp	0.33 ± 0.03 (a)	10.83 ± 1.02 (a)	0.20 ± 0.01 (a)	-	-	$0.997 \pm 2 \cdot 10^{-3}$	
	Mesotrione + S-metolachlor	DoseResp	0.43 ± 0.10 (a)	3.54 ± 0.64 (a)	0.31 ± 0.08 (a)	-	-	0.931 ± 0.02	
	Mesotrione + Nicosulfuron + S-metolachlor	DoseResp	0.43 ± 0.03 (a)	6.97 ± 0.23 (a)	0.19 ± 0.01 (a)	-	-	0.969 ± 0.01	
M3 ⁽²⁾	Mesotrione	DoseResp	0.65 ± 0.01 (c)	5.26 ± 0.46 (a)	0.22 ± 0.01 (b)	-	-	$0.985 \pm 4 \cdot 10^{-3}$	
	Mesotrione + Nicosulfuron	DoseResp	0.65 ± 0.01 (c)	14.16 ± 1.39 (b)	$0.14 \pm 3 \cdot 10^{-3}$ (a)	-	-	$0.999 \pm 2 \cdot 10^{-4}$	
	Mesotrione + S-metolachlor	DoseResp	0.58 ± 0.02 (b)	3.89 ± 0.40 (a)	0.21 ± 0.02 (b)	-	-	0.871 ± 0.03	
	Mesotrione + Nicosulfuron + S-metolachlor	DoseResp	0.38 ± 0.01 (a)	11.21 ± 0.11 (b)	0.14 ± 0.01 (a)	-	-	$0.998 \pm 1 \cdot 10^{-3}$	

⁽¹⁾: data for the second kinetic phase of the intermediate metabolites M2 and M3, (*i.e.* decrease of metabolite concentration)

⁽²⁾: data for the first kinetic phase of the intermediate metabolites M2 and M3 (*i.e.* increase of metabolite concentration)

k_1 , k_2 and p : rate constants. * p in $hour^{-1}$ (mesotrione) or day^{-1} (AMBA, M2, M3).

Table 3. Toxicity values of chemicals towards *A. fischeri* (Microtox®). Experimental data were compared to predicted toxicity values according to the Concentration Addition (CA) and the Independent Action (IA) models. The effect of the other components of the mixture on each compound were qualified of synergistic, additive and antagonistic according to MDR (Model Deviation Ratio) values (see Materials and Methods section). The results are reported as the mean \pm standard error (SE), n = 3.

Chemicals and mixtures (ratio)	Microtox® IC ₅₀ , 15 min (μM)			MDR (CA)	MDR (IA)	Effect (predicted by CA)	Effect (predicted by IA)
	Observed (experimental data)	Predicted (CA model)	Predicted (IA model)				
Mesotrione	128.5 \pm 7.1 ^a	-	-	-	-	-	-
Nicosulfuron	420.3 \pm 47.7 ^c	-	-	-	-	-	-
S-metolachlor	628.6 \pm 80.3 ^b	-	-	-	-	-	-
AMBA	82.7 \pm 18.6 ^a	-	-	-	-	-	-
ADMP	22.4 \pm 8.4 ^c	-	-	-	-	-	-
ASDM	> 3900 ^c	-	-	-	-	-	-
Mesotrione + nicosulfuron (1:1)	42.4 \pm 4.0	196.8 \pm 12.4	239.1 \pm 1.9	4.6	5.6	Synergistic	Synergistic
Mesotrione + S-metolachlor (1:1)	42.4 \pm 2.3	213.4 \pm 13.1	146.8 \pm 1.7	5.0	3.5	Synergistic	Synergistic
Nicosulfuron + S-metolachlor (1:1)	110 \pm 0.7	503.8 \pm 59.8	213.4 \pm 0.7	4.6	2	Synergistic	Synergistic
Mesotrione + nicosulfuron + S-metolachlor (1:1:1)	51.6 \pm 2.1	255.3 \pm 17.2	122.8 \pm 2.2	4.9	2.4	Synergistic	Synergistic
ADMP + ASDM (1:1)	391.3 \pm 25.2 ^c	44.5 \pm 1.8	24.1 \pm 0.4	0.1	0.1	Antagonistic	Antagonistic
AMBA + ADMP (0.31:1)	97.8 \pm 2.2 (AMBA)	27.1 \pm 9.7	24.8 \pm 0.7	0.3	0.3	Antagonistic	Antagonistic
AMBA + ASDM (0.31:1)	312.8 \pm 6.9 (ADMP)	327.1 \pm 1.3	82.6 \pm 0.3	0.1	0.1	Antagonistic	Antagonistic
	68.3 \pm 0.9 (AMBA)			4.8	1.2	Synergistic	Additive
	218.4 \pm 2.8 (ASDM)			1.5	0.4	Additive	Antagonistic
AMBA + ADMP + ASDM (0.31:1:1)	69.1 \pm 1.5 (AMBA)	47.5 \pm 2.1	24.8 \pm 0.7	0.7	0.4	Additive	Antagonistic
	221.2 \pm 4.9 (ADMP)			0.2	0.1	Antagonistic	Antagonistic
	221.2 \pm 4.9 (ASDM)			0.2	0.1	Antagonistic	Antagonistic
S-metolachlor + AMBA + ADMP (1:0.31:1)	147.6 \pm 8.5 (S-metolachlor)	46.2 \pm 15.6	14.3 \pm 0.9	0.3	0.1	Antagonistic	Antagonistic
	46.1 \pm 2.7 (AMBA)			1.0	0.3	Additive	Antagonistic
	147.6 \pm 8.5 (ADMP)			0.3	0.1	Antagonistic	Antagonistic
S-metolachlor + AMBA + ASDM (1:0.31:1)	135.6 \pm 5.8 (S-metolachlor)	412.8 \pm 2.2	150.4 \pm 0.6	3.0	1.1	Synergistic	Additive
	42.4 \pm 1.8 (AMBA)			9.7	3.6	Synergistic	Synergistic
	135.6 \pm 5.8 (ASDM)			3.0	1.1	Synergistic	Additive
S-metolachlor + AMBA + ADMP + ASDM (1:0.31:1:1)	213.6 \pm 20.3 (S-metolachlor)	65.9 \pm 2.9	14.3 \pm 0.9	0.3	0.1	Antagonistic	Antagonistic
	66.8 \pm 6.4 (AMBA)			1.0	0.2	Additive	Antagonistic
	213.6 \pm 20.3 (ADMP)			0.3	0.1	Antagonistic	Antagonistic
	213.6 \pm 20.3 (ASDM)			0.3	0.1	Antagonistic	Antagonistic

IC₅₀ = Inhibitory Concentration 50%; the IC₅₀ values obtained in the present study were similar to those found by ^a Bonnet et al. [7], ^b Joly et al. [28] and ^c Carles et al. [9]. The predicted (CA and IA) IC_{50, mix} values of mixtures containing ASDM were calculated with an IC₅₀ value of ASDM = 3900 μM.