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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

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HIGHLIGHTS

- Realistic maize herbicides mixtures were tested with Microtox® test.
- Formulated compounds are more toxic than active ingredients.
- High toxicity toward *Vibrio fischeri* occurs for recommended agricultural mixtures.
- A study to assess the use of chemicals as preconized by the REACH regulation.

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ABSTRACT

The Microtox® test, using the prokaryote *Vibrio fischeri,* was employed to assess the toxicity of the maize herbicides S-metolachlor, benoxacor, mesotrione and nicosulfuron, and their formulated compounds: Dual Gold Safeneur®, Callisto® and Milagro®; alone and in mixtures. For each compound we obtained original IC_{50} values, with consistent higher toxicities for formulated compounds compared to active ingredients alone. Mixtures of the four herbicides, prepared according to application doses encountered in agriculture, were found to be toxic at a lower concentration than single molecules. Mesotrione and nicosulfuron mixture appeared to be highly toxic to *V. fischeri,* however, this recommended post-emergence combination for maize crops got its toxicity decreased in formulated compound mixtures, suggesting that chemical interactions could potentially reduce the toxicity. Data comparisons to theoretical models showed a good prediction of mixture toxicity by Concentration Addition concept. Results seemed to exclude any synergistic effects on *V. fischeri* for the tested herbicide mixtures. Additional work coupling these bioassay data to ecosystemic level studies (aquatic and soil compartments) and data on additives and degradation products toxicity, will help to fill the gap in our knowledge of the environmental impact of these xenobiotics and in the choice of a more sustainable use of pesticides.

1. Introduction

Chemical risk assessment remains one of the major challenges to answer the problem raised by agriculture and especially intensive production. Herbicides are used in large amounts in agriculture but also in urban and domestic applications, and represent potential pollutants which need to be investigated. In 2011 in France, 62 700 tons of pesticides were sold, including a major part of herbicides.[2] These compounds are now well described, and some of them have the capacity to pollute aquatic ecosystems after their application and also lead to potential non-target effects (DeLorenzo et al., 2001; Fleeger et al., 2003).

To screen the potential toxicity of xenobiotics in aquatic ecosystems, different biotests have been developed. Among them, Microtox® is an *in vitro* test system which uses the bioluminescent bacteria *Vibrio fischeri* for the detection of pure chemicals, mixtures or natural samples toxicity (Bond and Martin, 2005). Therefore, significant interspecies correlations have been obtained for some classes of substances, between bacteria test data with *V. fischeri* and bioassay data for other organisms (Kaiser, 1998).

In 2003, atrazine (2-chloro-4-ethylamino-6-iso-propylamino-1,3,5-triazine), was banned due to its high toxicity on numerous organisms (Graymore et al., 2001) and also its persistence and its transfer capacities in environment. To replace atrazine, new

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selective herbicides have been developed and applied in mixtures in maize crops. In France, mesotrione, one of these recent selective herbicides, is often use in combination with S-metolachlor, benoxacor and nicosulfuron, to ensure maize crops yields. These molecules belong to different families and differ in their herbicidal mode of action (Table 1).

S-metolachlor is the active enantiomere of the metolachlor molecule, banned in 2003 in France. It is one of the best-selling herbicides for maize crops. It was developed by Ciba-Geigy (metolachlor) and marketed under the name Dual Gold® (Syngenta Agro S.A.S.). However, this first commercial product is not sold, neither authorized, in France.[3] S-metolachlor is now use in combination with a phytoprotector molecule named benoxacor and sold by Syngenta Agro S.A.S. under the marketed product Dual Gold Safeneur®.

Mesotrione is a selective pre- and post-emergence herbicide, which controls the growth of most broadleaf and some weed grass in maize crops. It was developed by Syngenta Agro S.A.S., marketed under the commercial name Callisto®. Increased toxicity of the commercial formulation compared to the single mesotrione molecule has already been found in *V. fischeri* bioassay (Bonnet et al., 2008). The metabolite 4-methylsulfonyl-2-nitrobenzoic acid (MNBA) and the 2-amino-4-methylsulfonyl-benzoic acid (AMBA), two degradation products of mesotrione, are found with the parent molecule in soil and water, and AMBA was found to be more toxic than the parent molecule to *V. fischeri* (Bonnet et al., 2008). Other metabolites have been described but no complete pathway for the total dissipation of mesotrione has been identified so far (Batisson et al., 2009; Durand et al., 2010).

Nicosulfuron has been developed by ISK Bioscience Corporation and is used as a selective post-emergence control herbicide in maize of annual grass, broad-leaved and perennial weeds. Nicosulfuron is marketed by Syngenta Agro S.A.S. under the commercial name Milagro®. Its toxicity has already been evaluated using freshwater microalgae (Leboulanger et al., 2001), but so far there is no toxicity data available on Milagro® toxicity to *V. fischeri*.

These four herbicides are classified by the GHS hazard statement as very toxic to aquatic life (H400) and very toxic to aquatic life with long lasting effects (H410) in the environmental hazards index. Extensive studies on separate compounds and mixture of such chemicals are a priority, as underlined by the REACH regulation, which addresses production and use of chemicals in the EU (The REACH baseline study: A tool to monitor the new EU policy on chemicals – REACH (Registration, Evaluation, Authorisation and restriction of Chemicals), 2009).

Despite the fact that numerous studies have been done on separate herbicide compounds in bioassays (Osano et al., 2002), or in environmental compartments (Lo, 2010), the current trend in agriculture is the use of herbicide mixtures, raising therefore the need to assess their toxicity. S-metolachlor is mainly used as a pre-emergence herbicide on maize and its action is often completed by post-emergence herbicides like mesotrione and nicosulfuron. By choosing these herbicides, we reproduced realistic conditions in terms of mixture ratios and application rates encountered in agriculture.

The aim of this study was to consider *V. fischeri,* used in the Microtox® test, as a model of a non-target environmental prokaryote, (i) to determine the potential toxicity of S-metolachlor, benoxacor, mesotrione and nicosulfuron, alone and in mixture; (ii) to determine the toxicity of the commercial herbicidal products Dual Gold Safeneur®, Callisto® and Milagro® (active ingredient formulated with various additives); and (iii) to measure the toxicity of a realistic mixture of these formulated compounds on this

Table 1
General data on herbicides used in this study.

Active ingredient	Family	IUPAC name	Mode of action	Commercial formulation (Syngenta®)	References
S-metolachlor	Chloroacetanilide	(*S*)-2-Chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(1-methoxypropan-2-yl)acetamide	Inhibits the very long chain fatty acid (VLCFA) formation, which interferes with normal cell development and inhibits both cell division and cell enlargement	Dual Gold Safeneur® (915 g L^{-1} S-metolachlor; 45 g L^{-1} benoxacor)	http://www.plantprotection.org/hrac/
Benoxacor	Dichloroacetamide	(*RS*)-4-dichloroacetyl-3,4-dihydro-3-methyl-2*H*-1,4-benzoxazine	Increases the glutathione S-transferase activity in maize crops, in order to protect the plant from potential injuries caused by S-metolachlor		Fuerst et al., 1993, Abu-Qare and Duncan, 2002
Mesotrione	Triketone	2-[4-(Methylsulfonyl)-2-nitrobenzoyl]cyclohexane-1,3-dione	Inhibits a critical enzyme, p-hydroxy-phenylpyruvate dioxygenase (HPPD), in carotenoid biosynthesis	Callisto® (100 g L^{-1} mesotrione)	Mitchell et al., 2001
Nicosulfuron	Sulfonylurea	2-[(4,6-dimethoxypyrimidin-2-yl)carbamoylsulfamoyl]-N,N-dimethylpyridine-3-carboxamide	Prevents synthesis of isoleucine, leucine, and valine by inhibition of acetolactate synthase (ALS), which results in cessation of cell division and plant growth	Milagro® (40 g L^{-1} nicosulfuron)	http://www.iskbc.com/

[3] http://www.e-phy.agriculture.gouv.fr/

Table 2
Syngenta® data of active ingredient and maximum dose applications.

Formulated compound (f.c.)	Active ingredient (a.i.)	a.i. per liter of f.c. (g)	Maximum doses (L ha^{-1})	a.i. per hectare (g)
Dual Gold Safeneur®	S-metolachlor	915	2.1	1921.5
	Benoxacor	45		94.5
Callisto®	Mesotrione	100	1.5	150
Milagro®	Nicosulfuron	40	1.5	60

microorganism and to compare it to theoretical toxicity models values, in order to evaluate potential synergistic toxic effects.

2. Materials and methods

2.1. Chemical compounds

S-metolachlor (33859; Pestanal; purity, 98.4%), benoxacor (46001; Pestanal; purity, 99.0%), mesotrione (33855; Pestanal; purity, 99.9%), and nicosulfuron (34210; Pestanal; purity, 99.8%) were purchased from Fluka Riedel-de-Haën (Buchs, SG, Switzerland). The commercial products Dual Gold Safeneur® (concentrated suspension containing 915 g of S-metolachlor and 45 g of benoxacor per liter), Callisto® (concentrated suspension containing 100 g of mesotrione per liter) and Milagro® (concentrated suspension containing 40 g of nicosulfuron per liter) were manufactured by Syngenta Crop Protection (Table 2). They were obtained from a regular agricultural supplier.

2.2. Mixtures ratios

Mixtures of formulated compounds were realized according to Syngenta recommendations for the maximum dose of application in maize cultures (Table 2). Ratios of active ingredients were kept (Table 3) for both active ingredients and formulated compound mixtures. By doing so, measured IC_{50} values (X mg L^{-1}) in Section 3 (see below), correspond to the sum of the relative part of each active ingredient contained in the mixture ($x_1 + \cdots + x_n$ mg L^{-1}). Tested concentrations of the active ingredients were prepared by dilutions of stock solutions, with a final amount in DMSO of less than 0.5% (v/v), which has no effect on *V. fischeri* (Bogaerts et al., 2001). The commercial products were diluted with distilled water.

2.3. Microtox® assays

The *V. fischeri* bioluminescence inhibition observed in the presence of xenobiotic was measured after three exposure times (5, 15, and 30 min). All the materials for analysis (test reagent, diluents, osmotic adjusting solution, and reconstitution solution) were supplied by Azur Environmental (Carlsbad, CA, USA). For each compound, four independent experiments were performed according to the normalized procedure with a Microbics M 500 toxicity analyzer coupled to a PC using 500 DOS software for Microtox® (AZUR-Environmental, 1996). The aim of this test was to determine the median inhibitory concentration (IC_{50}), which is the concentration required to induce a 50% decrease in bioluminescence compared with the untreated bacteria after 5, 15 and 30 min.

2.4. Mixture toxicity models

Well described models of Concentration Addition (CA) and Independent Action (IA) were used to predict expected toxicity values of the different mixtures (Berenbaum, 1985).

CA, the model presented as a general solution for mixture toxicity analysis, is expressed mathematically as

$$\sum_{i=1}^{n} \frac{c_i}{ECx_i} = 1$$

where n is the number of mixture components, ECx_i is the concentration of the ith mixture component that provokes x% effect when

Table 3
Ratios of each active ingredient (in percentage of total active ingredients) in formulated compounds and related active ingredient mixtures.

	S-metolachlor (%)	Benoxacor (%)	Mesotrione (%)	Nicosulfuron (%)
Dual Gold Safeneur®	95.31	4.69		
Dual Gold Safeneur® + Callisto®	88.71	4.36	6.93	
Dual Gold Safeneur® + Milagro®	92.56	4.55		2.89
Dual Gold Safeneur® + Callisto® + Milagro®	86.32	4.25	6.74	2.69
Callisto® + Milagro®			71.43	28.57

Table 4
Toxicity values of the different chemicals towards *V. fischeri*.

Active ingredients	Microtox® 15 min IC_{50} (mg L^{-1})		Formulated compounds
Atrazine	196.7 ± 28.5[a]		
S-metolachlor	178.4 ± 22.8		
Benoxacor	93.3 ± 7.3		
S-metolachlor + benoxacor	174.5 ± 20.1	20.5 ± 2.0	Dual Gold Safeneur®
Mesotrione	43.6 ± 2.4[b]	1.1 ± 0.1[b]	Callisto®
Nicosulfuron	167.8 ± 21.8	4.1 ± 0.7	Milagro®
S-metolachlor + benoxacor + mesotrione	157.9 ± 32.2	10.4 ± 1.4	Dual Gold Safeneur® + Callisto®
S-metolachlor + benoxacor + nicosulfuron	202.7 ± 20.3	22.1 ± 3.9	Dual Gold Safeneur® + Milagro®
S-metolachlor + benoxacor + mesotrione + nicosulfuron	119.8 ± 24.9	14.1 ± 3.7	Dual Gold Safeneur® + Callisto® + Milagro®
Mesotrione + nicosulfuron	61.4 ± 10.6	4.8 ± 1.2	Callisto® + Milagro®

IC_{50} = Inhibitory Concentration 50%.
[a] Bogaerts et al. (2001).
[b] Bonnet et al. (2008).

applied singly and c_i is the concentration of the respective component in the mixture. If at a total concentration of the mixture provoking x% effect, the sum of toxic units equals one, concentration addition holds. For that reason, the sum of toxic units has been frequently used as a measure for comparing the observed toxicity with the predictions made by concentration addition.

IA, the model based on the assumption that the compounds of a given mixture act independently in a statistical sense, is expressed mathematically as

$$E(c_{Mix}) = E(c_1 + \cdots + c_N) = 1 - \prod_{i=1}^{n}[1 - E(c_i)]$$

where $E(c_{Mix})$ denotes the predicted effect (scaled to 0–1) of an n-compound mixture, c_{Mix} is the total concentration in the mixture, c_i is the concentration of the ith compound, and $E(c_i)$ is the effect of that concentration if the compound is applied singly. As $E(c_{Mix})$ values are scaled to 0–1, multiplication by 10^2 gives direct expected IC values for the considered mixture concentration (IC scaled to 0–100).

In this study, for both models, the effect E refers to the bioluminescence inhibition of *V. fischeri*.

2.5. Statistical analyses

Toxicity comparisons were performed between active ingredients and related formulated compound mixtures IC_{50}, using the Student's t-test for two samples. One-way ANOVA analyses followed by Tückey post hoc test, were performed to compare toxicity values of active ingredients IC_{50} on one hand, and formulated compounds IC_{50} on another hand. All statistical analyses were done with $n = 4$ (with n = the number of measurements).

$E(c_i)$ values for IA model were obtained from logarithmic regression curves of single compounds toxicity, and used to calculate the concentration–response relationships.

3. Results

The IC_{50} toxicity values (in mg L^{-1} equivalent of active ingredient) obtained with the active ingredients and the formulated compounds are reported in Table 4. The values of IC_{50} obtained at 5, 15 and 30 min were similar in most cases (13 of 14 tests), thus we only reported results for 15 min measurements. Significant differences between active ingredients and related formulated compounds toxicity ($P < 0.01$) were consistently observed, using Student t-test analyses (Fig. 1).

3.1. Toxicity assessment of active ingredients

The technical grade S-metolachlor, benoxacor, mesotrione and nicosulfuron showed a non-negligible toxicity compared to atrazine molecule (Table 4, IC_{50} = 196.7 ± 28.5 mg L^{-1}). IC_{50} values were not statistically different for S-metolachlor (178.4 ± 22.8 mg L^{-1}) and nicosulfuron (167.8 ± 21.8 mg L^{-1}), and significantly lower for benoxacor (93.3 ± 7.3 mg L^{-1}) and mesotrione (43.6 ± 2.4 mg L^{-1}).

Benoxacor did not change the toxicity of S-metolachlor molecule with an IC_{50} for S-metolachlor + benoxacor (174.5 ± 20.1 mg L^{-1}) similar to the toxicity of S-metolachlor alone.

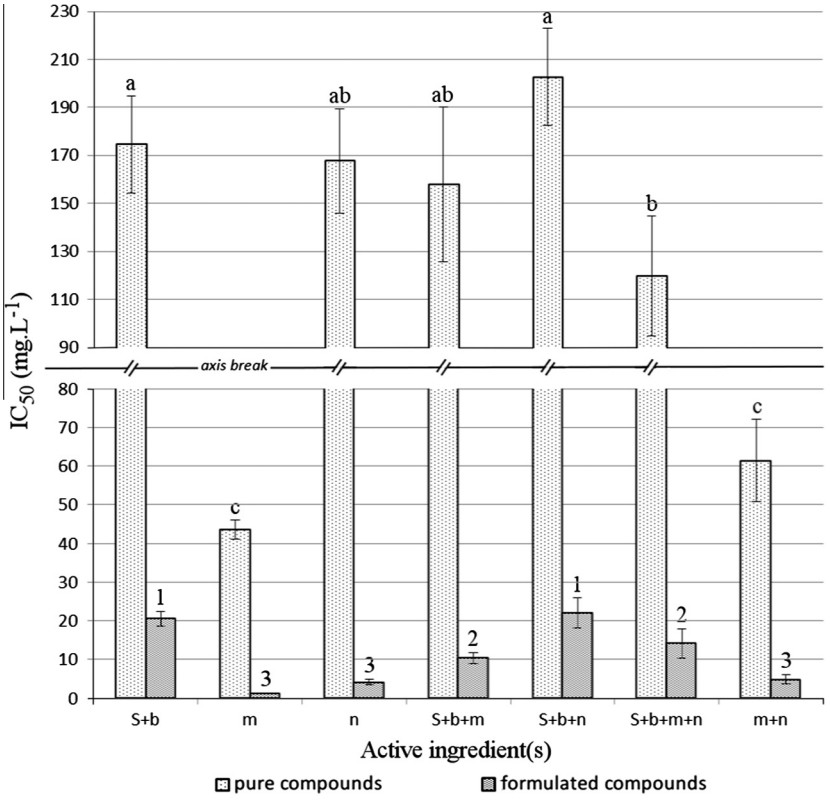

Fig. 1. IC_{50} values (mg L^{-1} of active ingredients content), for pure compounds (light gray) and formulated compounds (dark gray), used alone or in mixture. Herbicides: S-metolachlor (S), benoxacor (b), mesotrione (m) and nicosulfuron (n). Significant differences between pure compounds toxicity (labeled by a, b and c letters, P value: 9.164×10^{-9}) and formulated compounds toxicity (labeled by 1, 2 and 3 numbers, P value: 6.54×10^{-11}) were observed using one-way ANOVA analyses followed by Tückey post hoc test.

Table 5
Predicted toxicity values according to Concentration Addition (CA) and Independant Action (IA) models.

Mixture	Experimental data	Concentration Addition (CA)		Independant Action (IA)
	IC_{50} (mg L^{-1})	Raw CA values for IC_{50}[a]	Expected IC_{50} values (mg L^{-1})[b]	Expected inhibitory concentration (IC)[c]
Active ingredients				
S-metolachlor + benoxacor	174.5 ± 20.1	1.02	171.1 ± 19.7	$IC_{[52-65]}$
S-metolachlor + benoxacor + mesotrione	157.9 ± 32.2	1.11	142.3 ± 29.0	$IC_{[51-77]}$
S-metolachlor + benoxacor + nicosulfuron	202.7 ± 20.3	1.19	171.0 ± 17.1	$IC_{[56-68]}$
S-metolachlor + benoxacor + mesotrione + nicosulfuron	119.8 ± 24.9	0.84	142.9 ± 29.7	$IC_{[43-65]}$
Mesotrione + nicosulfuron	61.4 ± 10.6	1.11	55.3 ± 9.5	$IC_{[44-62]}$
Formulated compounds				
Dual Gold Safeneur® + Callisto®	10.4 ± 1.4	1.13	9.2 ± 1.2	$IC_{[57-74]}$
Dual Gold Safeneur® + Milagro®	22.1 ± 3.9	1.2	18.4 ± 3.2	$IC_{[49-70]}$
Dual Gold Safeneur® + Callisto® + Milagro®	14.1 ± 3.7	1.58	8.9 ± 2.3	$IC_{[53-91]}$
Callisto® + Milagro®	4.8 ± 1.2	3.45	1.4 ± 0.3	$IC_{[60-100]}$

[a] Direct results obtained from CA model calculation.
[b] Expected results obtained by applying raw CA value coefficient on experimental data.
[c] Expected results obtained from IA model calculation, including confident interval values.

Mesotrione showed the highest toxicity alone (43.6 ± 2.4 mg L^{-1}) and in presence of nicosulfuron, (61.4 ± 10.6 mg L^{-1}).

The IC_{50} evolution (Fig. 1) showed the toxic cumulative effect of the mixture (applied following Table 3 ratios) of the four active ingredients (i.e. S + b + m + n), compared to the mixtures S + b and S + b + n.

3.2. Toxicity assessment of formulated compounds

All of the formulated compounds presented toxicity increased by 8.5 times for Dual Gold Safeneur®, 39.6 times for Callisto® and 40.9 times for Milagro®, compared to their respective active ingredients (Table 4).

The toxicity of Dual Gold Safeneur® (20.5 ± 2.0 mg L^{-1}) is equivalent to the toxicity of the Dual Gold Safeneur® + Milagro® mixture (22.1 ± 3.9 mg L^{-1}), whereas the mixtures including Callisto® increased significantly the toxicity (10.4 ± 1.4 mg L^{-1} for Dual Gold Safeneur® + Callisto® mixture and 14.1 ± 3.7 mg L^{-1} for the three formulated compound mixtures).

Considering Table 3 ratios, Callisto®, the most toxic formulated compound (1.1 ± 0.1 mg L^{-1}) when used alone, shows a decreased toxicity when combined with Milagro®, with a total IC_{50} of 4.8 ± 1.2 mg L^{-1}. However, this result is non-significant according to one-way ANOVA analysis.

3.3. Predictability of mixture toxicity

Results of predicted toxicity according to CA and IA models are presented Table 5.

CA model predicted IC_{50} values accurately for all mixtures excepted for Callisto® + Milagro®, where the expected toxicity was more than 3 times higher than the experimental data (1.4 ± 0.3 mg L^{-1} vs. 4.8 ± 1.2 mg L^{-1}).

IA model was used to obtain expected IC values from the concentration–response logarithmic regression curves of single compounds, accordingly to Table 3 ratios. Compared to experimental IC_{50} values, IA appeared to generally over-estimate the IC of the mixtures (except for S-metolachlor + benoxacor + mesotrione + nicosulfuron mixture, mesotrione + nicosulfuron mixture and Dual Gold Safeneur® + Milagro® mixture, where IC scaled respectively from 43 to 65, 44 to 62 and 49 to 70).

4. Discussion

This work presents original data concerning the potential toxicity of S-metolachlor, benoxacor, mesotrione and nicosulfuron, four herbicides used in maize cultures, towards *V. fischeri*, a model organism in ecotoxicology bioassays. To our knowledge, no comparable assessment of toxicity, with realistic field mixtures ratios, has been done on both active ingredients and formulated compounds (Deneer, 2000).

To evaluate toxicity of chemicals, it is necessary to assess their effects on different communities which can play a key role in ecosystem functions, as prokaryotic communities. This underlines the choice of Microtox® assay, using *V. fischeri*.

As Microtox® assay cannot take into account interaction between microorganisms occurring at the community level, toxicity results presented thereafter will not reflect realistic impact of pollutants in environment. However, Microtox® assay provides a useful technique for the determination and the comparison of the potential toxicity of various contaminants as well as for the assessment of toxicity interactions.

We did not neutralized the pH of both active ingredients and formulated compounds solutions as it has been observed that *V. fischeri* bioluminescence occurs between pH 4.5 and 10.5 (Scheerer

et al., 2006). Moreover, pH lower than 9.7 (as in this study) do not decrease the bioluminescence of *V. fischeri* (data not shown).

The commercial products Dual Gold Safeneur®, Callisto® and Milagro® have a higher toxicity compare to their corresponding active ingredients. We did not have any detailed information on additives added in formulated compounds but we can link them as major actors responsible for the acute toxicity observed. Nevertheless, safety data are now available for Callisto®[4] and describe two alcohol additives in the formulation, the poly(oxy-1,2-ethanediyl), alphaisodecyl-omegahydroxy- and the octan-1-ol, present respectively at 20–30% and 5–10% (w/w). Octan-1-ol IC_{50} is reported on *V. fischeri* at 46.2 mg L^{-1} (Villa et al., 2012). Its high toxicity associated to mesotrione and other adjuvants toxicities could explain the high IC_{50} value obtained for Callisto® (1.1 ± 0.1 mg L^{-1}, Table 4). Additives molecules are different among the formulated compounds and intensive studies should be carried out as soon as their identity will be revealed.

As a main result of this study, the very low IC_{50} observed for the mixture of mesotrione and nicosulfuron raised potential questions on the use of these two chemicals together as post-emergence herbicides, as it is actually recommended. However, this effect seems to be minimized in the mixture of formulated compounds (Callisto® + Milagro®) with an increase of the mesotrione concentration responsible for the IC_{50} (1.1 ± 0.1 mg L^{-1} alone, in contrast with 3.4 ± 0.8 mg L^{-1} in m + n mixture, for mesotrione active ingredient toxicity part, obtained from Table 3 ratios). This surprising result of decrease of the mixture toxicity could be due, in that specific case, to interactions between active ingredients and/or additives contained in the both formulated compounds (i.e. chelation, sequestration, etc.). These results lead to potential interactions hypotheses, which could be interesting to study, in parallel to other herbicides parameters (i.e. transfer properties, toxicity of degradation products, impact on soil/water communities, impact on biochemical cycles etc.), in order to minimize the toxicity of molecules applied alone by coupling them with other molecules able to reduce their global toxicity for environment, while taking in account the agricultural necessities.

Ecotoxicology studies on the impact of herbicides mixtures on soil system have been done in our research team (Joly et al., 2012) and further analysis using the same mixtures are in progress.

This study underlined the strength of CA model (Table 5) to predict the mixtures toxicity, despite the fact that the compounds have different molecular acceptor sites (Table 1) and may act on different physiological systems within *V. fischeri*, which suppose a better prediction by IA model. Nevertheless, both models overestimated the Callisto® + Milagro® mixture toxicity (Table 5), which confirm the usefulness of the experiment to corroborate models predictions.

However, our study, in accordance with other studies using these models (Berenbaum, 1985; Altenburger et al., 2000; Faust et al., 2003; Villa et al., 2012), assumes the fact that CA model may be considered as a pragmatic and realistically acceptable worst case, capable to ensure an adequate level of protection. In so, synergistic effects seemed to be excluded for the tested mixtures.

To draw a more detailed picture of the impact and fate of these four herbicides on the environment, it would be necessary to perform bioassays similar to the Microtox® test on all newly identified degradation products. Indeed the high toxicity values presented in this work could be even higher for *V. fischeri* with degradation products, as it has been observed with mesotrione and its metabolite AMBA (Bonnet et al., 2008) or with 2-ethyl-6-methylamine, a metabolite of metolachlor (Osano et al., 2002). Another issue could be the use of extracted and purified active ingredients and degradation products from contaminated soil or slurry, to test them as a true exposition mixture.

The understanding of the impact of herbicides mixtures, leads to large studies in term of models, from bioassay to field experiments, by dissociating the molecules responsible for the toxicity (active ingredients, degradation products and additives), and by taking in account the application ratios encountered in agriculture or found in receptor ecosystems. In that way, new automated tools for *in situ* aquatic toxicity determination using *V. fischeri* are currently developed and can be used on-line to obtain automatic alerts on abnormal concentrations of toxic compounds (Lopez-Roldan et al., 2012). Further investigations will be necessary and are still in progress to fill the gap in our knowledge of the environmental impact of these xenobiotics, in order to help in the choice of a more reasoned use of pesticides.

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