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TOXICITY OF SULCOTRIONE PHOTOPRODUCTS MIXTURE TOWARDS *VIBRIO FISCHERI* IN THE AQUATIC ENVIRONMENT

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Abstract: Photodegradation by sunlight radiation is one of the most destructive pathways for pesticides after their application in the field. The generated photoproducts can exhibit various toxicological properties and affect non–target organisms. Sulcotrione is a herbicide believed to be a relatively non–toxic alternative to atrazine herbicides used on corn fields. Despite many tests required for placing plant protection products on the market, it still happens that transformation pathway and the toxicological profile of these compounds is not fully understood. The results presented in this article are complementary to the research performed by a research group from National Center for Scientific Research (CNRS) at the University of Blaise Pascal (Auvergne, France). Sulcotrione is one of main herbicides used to protect the maize plantations in the region of Auvergne (France), as well as in Poland. As part of the experiments, the distribution of sulcotrione under the influence of polychromatic radiation (fluorescent lamp, $\lambda > 295$ nm, suitable for environmental tests) in aqueous solution of pH 6.5 was tested. The main products of these reactions were 1H–xanthene–1,9–dione–3,4–dihydro–6–methylsulfonyl (CP) and 2-chloro-4-methylsulfonyl-benzoic acid (CMBA), which are the result of intra-molecular cyclization and hydrolysis of sulcotrione, respectively. These products were quantified by using HPLC-diode array detector analysis. The studies clearly show an increase in toxicity towards tested organism (*Vibrio fischeri bacteria*) with the increase of irradiation time and appearance of the photoproducts. The results suggest that the observed increase in toxicity may be rather attributed to the occurrence of the same minor photoproducts than to the presence of the major photoproducts (CP and CMBA). Identification of the minor photoproducts could not be performed using the current instrumental equipment.

INTRODUCTION

Sunlight radiation is one of the most important abiotic factors responsible for the environmental fate of pesticides, which were applied to plants (leaves) and into the soil during agrotechnical procedures [10]. Photodegradation processes also significantly influence the fate of pesticides in surface waters. The photochemical reactions can proceed due to direct absorption of solar radiation by molecules in the actinic spectrum or/and via indirect (photoinduced) reactions mediated by humic substances or nitrites [4]. Losses of active molecules due to photodegradation can lower the treatment efficiency towards

target weeds [18]. On the other hand, the photoproducts of herbicides can exhibit various toxicological properties towards non–target organisms, i.e. (i) the bacterial (*V. Fischeri*) toxicity of aqueous trifluralin herbicide solutions decreased with increasing irradiation time [4], but in contrast, (ii) an increase in toxicity of some acifluorfen and phenylurea herbicide photoproducts was reported, after irradiation, using non–target aquatic organisms such as *Daphnia magna* [13] and *Vibrio fischeri* [1], respectively.

Sulcotrione (2–[2–chloro–4–(methylsulfonyl)benzoyl]–1,3–cyclohexanedione) is a post–emergent herbicide widely used in agriculture against grass and broad–leaved weeds. This herbicide is an alternative to glyphosate and atrazine [3], the latter being prohibited from use in all the EU member states since 2007 [6].

Little information is available concerning the photochemistry of sulcotrione and the effect of its photoproducts on non–target microorganisms. The first reported study on sulcotrione phototransformation was published by Ter Halle *et al*. [15]. Generation of the cyclization product CP (1H–xanthene–1,9–dione–3,4–dihydro–6–methylsulfonyl) under solar-simulating conditions was confirmed on the cuticular wax coating of the leaves' surface at lab scale as well as in environmental studies in corn fields after spraying [16]. CP is also a main photopoduct in the aquatic medium [15, 17].

Chaabane *et al.* [2] reported the qualitative analysis of photoproducts formed under UV radiation and the toxicity of an irradiated solution of sulcotrione towards some heterotrophic marine bacteria and cyanobacteria. However, no reference was made to the major CP product detected in the previous studies by Ter Halle *et al.* [15].

While the toxicological studies on individual (main) photoproducts have already been carried out [17], the toxicological effect of a mixture of photoproducts needs to be clarified. The aim of this study is to provide complementary information on the evolution of toxicity of sulcotrione water solution during the irradiation with formed UV light.

MATERIALS AND METHODS

Chemicals and materials

Sulcotrione (S) (98.7%) (Riedel de Haen), 2–chloro–4–methylsulphonylbenzoic acid (CMBA) (95%, ACROS), and xanthene-1,9-dione-3,4- dihydro-6-methylsulfonyl (CP) (synthesized and purified according to the procedure of Ter Halle *et al.* [14]) were used in the studies.

The methylene chloride (gradient grade, Riedel de Haen), acetonitrile (HPLC grade, Riedel de Haen) and distilled water (milli–Q, Millipore) were used as solvents. Na_2HPO_4 (99%) and K_2HPO_4 (99.5% , PROLABO) were used as buffers.

Photochemistry of sulcotrione in water

An aqueous solution of sulcotrione (100 mg L^{-1}, 3.14 x 10^{-4} M) was adjusted to pH 6.5 by adding phosphate buffer (10^{-1} M). Photochemical experiments (n = 4) were carried out in Pyrex tube reactors ($\lambda > 295$ nm, vol. 20 ml) operating in batch test mode. The irradiation device was made of six tubes of 20 W sunlamps (Ducke FL 20) fitted in a metal cylindrical enclosure. The lamps and the reactor were respectively placed along the focal axes of a mirror. The fluorescent lamps emitted radiation in the range from 270 to 600 nm wavelength with maximum radiation at 313 nm wavelength (Fig. 2b). The cooling fan installed at the bottom allowed for a stable temperature of 20°C to be maintained in the reactor.

The chemical yield of a product was defined by the following Equation (1):

$$Yield\ (P) = \frac{[P]t}{[S]o} \times 100 \quad (1)$$

where $[P]_t$ is the molar concentration of a photoproduct at different irradiation times (t), $[S]_o$ sulcotrione initial molar concentration.

Dark control experiments showed no loss of compounds by adsorption on glass surfaces or by spontaneous transformation during a period of time corresponding to the duration of irradiation experiments.

Analytical equipment

HPLC–diode array detector analyses (HP1050) were performed at room temperature using reverse phase column (Colonne ZORBAX SB–CN, Agilent, 4.6 x 250 mm, 5 µm). The mobile phase was a mixture of acetonitrile (A) and water (B) (acidified with formic acid, 3‰ v/v). A flow rate of 1 mL min^{-1} was used for all the analyses. The linear gradient for HPLC analysis was as follows: the initial composition, A/B 16/84 v/v%, was held for 10 min and then was changed to A/B 40/60 v/v% over a period of 10 min and maintained for an additional 5 min, then changed to A/B 16/84 v/v% for 5 min and finally was held for 10 min at A/B 16/84 v/v%. The TOC values at different irradiation times were determined using TOC Analyzer, TOC–5050–A (Shimadzu).

Toxicity measurements

The toxicity of an irradiated solution of sulcotrione was determined using *Vibrio fischeri* bacteria. The Microtox® system consisted of lyophilized bacterial reagent *V. fischeri*, reconstitution reagents and the Model 500 Toxicity Analyzer (AZUR Environmental). A 2% NaCl solution (Microtox® diluent) was used as diluent. The samples from different irradiation times were adjusted to pH 7 by adding phosphate buffer solution into each sample. Toxicity was expressed in toxicity units (TU) based on the EC_{50} value, which is the concentration that causes 50% reduction of bioluminescence after 30 min of incubation. The EC_{50} value used in calculations was the average value of 4 replicates. Moreover, the toxicity for solution containing two main photoproducts (280 mg L^{-1} of CP and 280 mg L^{-1} of CMBA) was also evaluated.

The empirical toxicity unit ($TU_{empirical}$) was calculated as follows (Equation 2):

$$TU_{empirical} = \frac{1}{F_{EC50}} \quad (2)$$

where: F_{EC50} is a dilution factor of the tested sample (dilution of the original or irradiated solutions) in order to obtain 50% reduction of bioluminescence (EC_{50} value), i.e. if F_{EC50} equal to 0.5, the $TU_{empirical}$ equal to 2.

Then a theoretical toxicity unit ($TU_{theoritical}$) was calculated for irradiated solution mixtures as the sum of the TU contribution of each component according to Rigol *et al.* [13] (Equation 3).

$$TU_{theoretical} = \sum_{i=1}^{n} \frac{C_i}{EC_{50i}} = \sum TU_i \quad (3)$$

where: n is the number of mixture components, C_i is the concentration (in mg L^{-1}) of S, CP and CMBA (the parent compound and main photoproducts detected by HPLC–diode array detector) in the mixture, and EC_{50i} is the concentration of the respective components (quantified by HPLC analysis) that cause 50% reduction of bioluminescence.

RESULTS AND DISCUSSION

Photoproducts of sulcotrione in water

The photochemical removal rate (pseudo–first order kinetic $R^2 = 0.96$ with $k = 0.0589\ h^{-1}$) determined in these studies cannot be compared directly with previous studies because of differing volumes of irradiated solutions, light intensity, or emission lamp spectra. However, irrespective of the system used, i.e. xenon lamps (sunlight simulator $\lambda > 290$ nm),"black light" lamps (94% of radiant energy at 365 nm) [15] or fluorescent lamps (present studies), the distribution of the main photoproducts (Fig. 1) was not influenced.

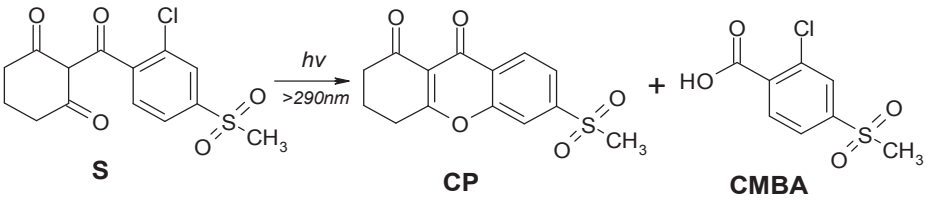

Figure 1. Main photoproducts of sulcotrione photolysis in water

Apparently, the main products of the photochemical reaction were: cyclization product (CP) formed at the beginning of the reaction, followed by hydrolysis product of sulcotrion (CMBA) (Figure 1, Figure 2).

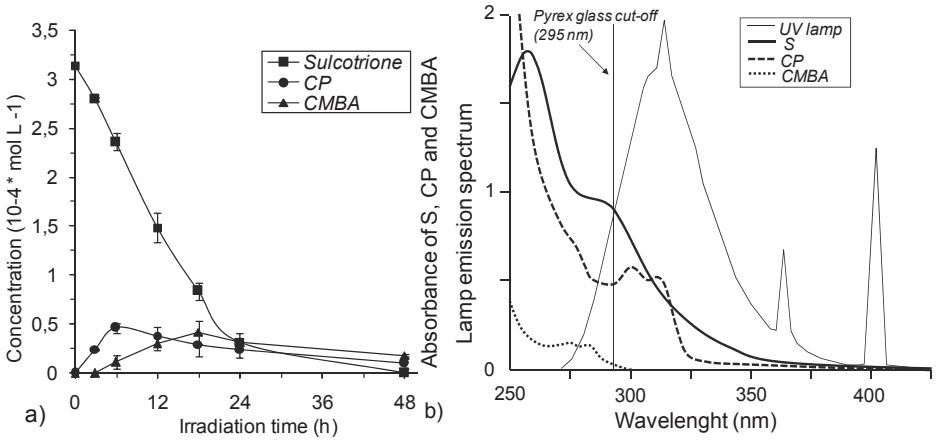

Figure 2. Phototransformation of sulcotrione and formation of CP and CMBA, analyzed by HPLC– diode array detector (a), Absorption spectra in water (10^{-4} M, pH 6.5), sulcotrione (S), main photoproducts CP and CMBA and emission spectrum of the adopted UV lamp (Ducke FL 20) (b)

Chemical yield calculated for CP reached 15% within 3h of irradiation which corresponded to 25% of the extent of sulcotrione conversion. The maximum of 13% of CMBA occurrence was observed within 18 h of irradiation and at a sulcotrione conversion extent of 73%. Both breakdown products, CP and CMBA, are detected at amounts representing

≥ 10% of the active substance added (sulcotrione), which is a threshold value. Consequently such products are considered as "major metabolites" and according to registration requirements [7], their fate and behavior in the environment need to be determined. However, according to the EFSA Scientific Report [13] only CMBA is recognized as the main photoproduct of sulcotrione (with a maximal chemical yield of 27%)

Chaanabe *et al.* [2] did not observe CP during photodegradation of sulcotrione in various aquatic environments. Most probably distribution of the breakdown products of photochemical reaction was different, because of a propagation of higher energy, shorter wavelengths (i.e. UVC) in the quartz glass reactor used in the mentioned experiments [2]. The authors obtained a three–fold greater degradation rate of sulcotrione in the quartz reactor irradiated by lamps emitting at 280–700 nm. As a consequence, the CP could easily be further photodegraded and thus become undetectable. In contrast the more simple molecules, such as the cyclohexanedione (CHD) found by Chaanabe *et al.* [2] as a photoproduct of sulcotrione degradation, were not detected in the experiments presented (Figure 3). The standard solution of CHD injected into the column should give a peak at retention time 4.9 min (data not shown).

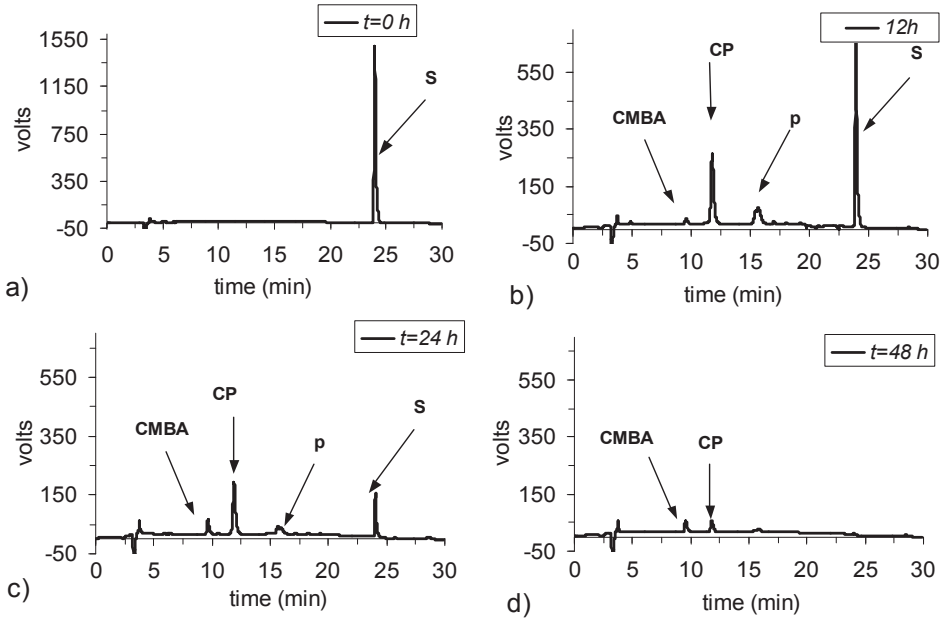

Figure 3. HPLC chromatogram (at 240 nm) of an irradiated aqueous solution of sulcotrione (pH 6.5). Sulcotrione conversion percentage: 0% (a), 47% (b), 90 % (c) and 100 % (d)

These experiments were carried out in a reactor made of Pyrex, the material which attains a maximum transmission level at 340 nm and beyond. These conditions exclude radiation at wavelengths $\lambda < 295$ nm [11], and comply well with the requirements for the stability test in water imposed by the Plant Protection Products Directive [7]. It should be stressed that identification of the CP product was performed by isolation of the molecule and structural determination by 1H and 13C NMR, and confirmed by HPLC/MS (ESI + $m/z = 293$; ESI - $m/z = 291$) [8].

Toxicity of irradiated solutions of sulcotrione and TOC removal

Toxicity was measured using the Microtox® test, a standard test widely used as a non–specific indicator of substance toxicity for monitoring purposes in environmental and industrial wastewater samples [12].

Because of the low toxicity of sulcotrione, the EC_{50} for sulcotrione was 374 mg L^{-1} [16]; the solution of sulcotrione before irradiation (103 mg L^{-1}) did not show any measurable acute toxicity for *V. fischeri*. However, it could be calculated that the $TU_{theoretical}$ of the initial solution equaled 0.28.

In the studies the empirical TU was always greater than the theoretical TU, which indicates that a synergism among components is produced [13] or/and unknown intermediates (not identified in the present studied) highly contribute to the overall toxicity (Figure 4a). As it was shown in the previous studies [17], main photoproduct CP (EC_{50} = 42 mg L^{-1}), is 9–fold more toxic than the parent substance, while the CMBA (EC_{50} = 314 mg L^{-1}) is only slightly more toxic than sulcotrione. The theoretical TU determined for irradiation solution increased, but never exceeded the value of 1 TU (Figure 4a).

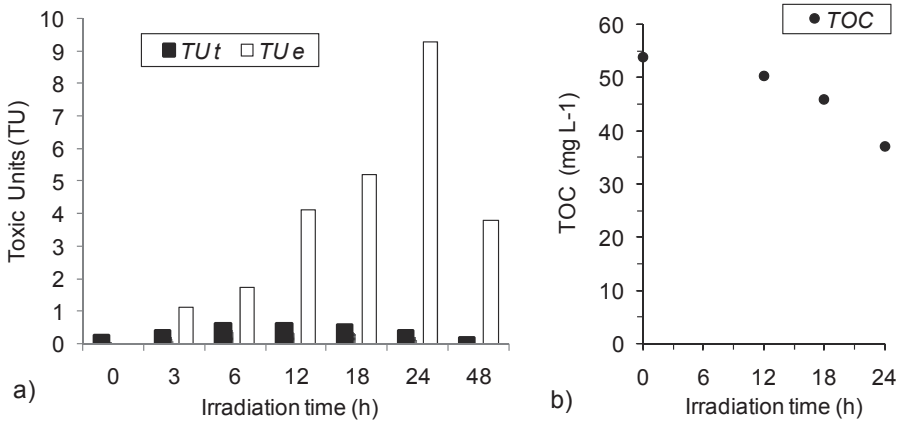

Figure 4. Evolution of toxicity (a) and TOC in an irradiated solution of sulcotrione (b)

Synergism among two main photoproducts CP and CMBA can be rather excluded. Mixture toxicity test performed for solution containing 280 mg L^{-1} of each photoproduct showed that the empirical TU (6.0) was lower than theoretical TU (7.6). It indicates that interaction of the two chemicals produce an effect lower than the additive effect.

On the other hand, a significant increase of the empirical TU was already observed within 3h of irradiation (Figure 4a). A maximum toxic response ($TU_{empirical}$) (towards *V. fischeri*) was observed after 24h of irradiation (Figure 4a), when 90% of sulcotrione was converted, but the major photoproducts, CP and CMBA, started to decline considerably (Figure 2). It is conceivable that the increase of toxicity was due to some minor photoproducts present in the reaction mixture (retention time at 15.5 min) (Figure 4b–d), which it is impossible to quantify without sample pretreatment (good separation, isolation and concentration).

The prolonged reaction time resulted in a decrease of toxicity, but nevertheless within 48h of irradiation the treated solution showed much higher toxicity (TU = 4) than initially.

A sulcotrione conversion rate of 50% was reached within 12 h of irradiation, but only moderate evolution of TOC (Fig. 4b) suggested that mineralization of this compound was rather limited in that period. The evolution can be correlated with the increased concentration of CP followed by formation of CMBA (Fig. 2). A much more rapid decline of TOC after 18 h, when the photochemical reaction was allowed to run that far, can certainly be attributed to the oxidation of organic compounds into CO_2, water etc. Within 24 h of irradiation mineralization leveled at 31%.

The only toxicity assessment of sulcotrione previously reported by Chaanabe *et al.* [2], showed that μg L^{-1} levels of irradiated solutions of sulcotrione were unlikely to harm the tested aquatic species. The concentration of herbicide (amounted to 100 mg L^{-1}) used in the experimental approach was far from the environmentally realistic level, which for instance is in the range 10–25 ng L^{-1} in Lake Greifensee in Switzerland, as reported by Freitas *et al.* [8]. Nevertheless, owing to punctual contamination after spraying of herbicides much higher concentrations would be expected in the close vicinity of the field in question. Especially, seasonal application of pesticides cause an excessive penetration of these agro-chemical pollutants into the surface water [9]. The main threat of sulcotrione would be rather attributed to its seasonal massive application and its mobility (in soil Kd value on average 0.81 mL g^{-1} [5]) then its persistence in the environment (moderate single first order DT_{50} in soil were in the range 10.8–89.7 days [5]).

CONCLUSION

Photochemical reaction significantly contributes to the fate of sulcotrione in the environment (including surface waters). Photodegradation of herbicides due to sunlight results in a greater demand of herbicides to be introduced into the environment in order to ensure the plant protection activity.

The toxicological studies using *V. fischeri* (Microtox® test) indicated that some products can be more toxic than the parent substance. The reason for the significant rise of toxicity of irradiated sulcotrione are probably some minor photoproducts present in the reaction mixture, but not identified under studies. The toxicity of irradiation solutions of sulcotrione deserves closer examination using other organisms, representing different trophic levels in order to better estimate the possibility of perturbations caused by photoproducts for non–target organisms in aquatic and terrestrial ecosystems.

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TOKSYCZNOŚĆ FOTOCHEMICZNYCH PRODUKTÓW PRZEMIAN SULKOTRIONU W STOSUNKU DO *VIBRIO FISCHERI* W ŚRODOWISKU WODNYM

Fotodegradacja pod wpływem promieniowania słonecznego jest jednym z najbardziej destrukcyjnych procesów zachodzących w środowisku po aplikacji herbicydów na polu. Wytworzone fotoprodukty mogą wykazywać bardzo zróżnicowany profil toksykologiczny i wpływać na organizmy, które nie są celem działania herbicydu. Sulkotrion należy do rodziny herbicydów trójketonowych i jest uważany za nietoksyczną alternatywę dla herbicydów triazynowych. Pomimo wielu testów, jakim musi być poddany produkt ochrony roślin przed jego wprowadzeniem na rynek, nadal zdarza się, iż drogi przemian tych związków oraz profile toksykologiczne nie są w pełni poznane. Wyniki badań prezentowanych w niniejszym artykule stanowią uzupełnienie badań prowadzących przez grupę badaczy z Narodowego Centrum badań Naukowych (CNRS) przy Uniwersytecie Blaise Pascal w regionie Owernii (Francja). Sulkotrion jest jednym z głównych herbicydów używanych do ochrony plantacji kukurydzy w regionie Owerii (Francja) i jest on również stosowany w Polsce.
W ramach przeprowadzonych eksperymentów badano rozkład sulkotrionu pod wpływem promieniowania polichromatycznego (lampa fluorescencyjna, $\lambda > 295$ nm, odpowiednich dla testów środowiskowych) w roztworze wodnym o odczynie wynoszącym pH 6,5. Głównymi produktami tych przemian są 1H-ksanten-1,9-dionu-3,4-dihydro-6-metylosulfonylowy (CP) oraz kwas 2-chloro-4-metylosulfonylo-benzoesowy (CMBA), które są odpowiednio wynikiem wewnątrz cząsteczkowej cyklizacji oraz produktem hydrolizy sulkotrionu. Obecność tych produktów analizowano za pomocą układu chromatograficznego HPLC – z detektorem z matrycą fotodiodową. Przeprowadzone badania jednoznacznie wykazały wzrost toksyczności mieszaniny produktów

fotochemicznego rozkładu sulkotrionu w stosunku do testowanego organizmu (bakterii *Vibrio fischeri*). Wyniki sugerują, iż obserwowany wzrost toksyczności może być wywołany produktami przemian fototochemicznych innymi niż zidentyfikowane jako główne produkty przemian (CP i CMBA). Identyfikacja produktów pośrednich nie mogła być wykonana za pomocą użytej aparatury badawczej.