

## COMPARATIVE EVALUATION OF VETERINARY ACTIVE INGREDIENTS AND FORMULATIONS

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### Abstract

Chemical substances used in various fields of agriculture (e.g., veterinary medicine or crop protection) represent relevant environmental loads, and their residues, metabolites and decomposition products possibly occur in wastewater and can easily reach surface water. Adjuvants (e.g., surfactants) and other co-formulants used in veterinary medicine, feed additives, as well as in pesticide formulations have long been classified as inactive ingredients (AIs) in the aspects of the required main biological effect of the pharmaceutical or pesticide product. In wastewater management the application of the advanced oxidation processes (AOP) are in the focus of interest due to their high efficiency in the removal of persistent organic pollutants and pharmaceutical residues. To compare the toxicity of various AIs and formulations used in veterinary medicine, acute toxicity tests were performed on *Daphnia magna*. Additionally, effects of the presence of H<sub>2</sub>O<sub>2</sub> due to AOP on the toxicity of 0.1 mmol dm<sup>-3</sup> sulphamethoxazole (SMX) solutions oxidised during gamma irradiation (1 kGy, 2.5 kGy) were assessed. Ecotoxicological evaluation of the treated SMX solutions was carried out using three test organisms (*Vibrio fischeri*, *Pseudokircheriella subcapitata*, *D. magna*). Results showed significant differences in the individual acute toxicity of various veterinary AIs and formulations on *D. magna*. SMX and trimethoprim (TRI) were the least toxic investigated AIs; their evaluated EC<sub>50</sub> values were 98.06±58.67 and 93.06±33.17 mg L<sup>-1</sup>, respectively. The most toxic AI was sulphaguanidine (SGD) (EC<sub>50</sub> = 1,79±0.34 mg L<sup>-1</sup>). Significant differences were observed in the toxicity of the investigated veterinary drugs containing SMX and TRI. Their formulated veterinary pharmaceutical product SUMETROLIM was more toxic on *D. magna* (EC<sub>50</sub> = 106.17±54.86 mg L<sup>-1</sup>) compared to the COTRIUM-E. Combined toxicity was the highest when SMX and TRI were investigated together in SUMETROLIM equivalent concentrations compared to the formulated veterinary products. The untreated SMX solution resulted in 5±1% inhibition on *V. fischeri*, while higher, 30±2% inhibitions were detected in irradiated solutions due to the presence of H<sub>2</sub>O<sub>2</sub>. H<sub>2</sub>O<sub>2</sub> showed significantly high inhibition on the investigated test organisms. By the reduction of H<sub>2</sub>O<sub>2</sub> concentrations, decreased inhibition was observed on *V. fischeri* and *P. subcapitata*. The evaluated EC<sub>50</sub> for *V. fischeri*, *P. subcapitata* and *D. magna* were 0.349, 0.251 and 0.064 mmol dm<sup>-3</sup>, respectively.

### Introduction

Several chemical substances and their formulations are used in various fields of agriculture, such as veterinary medicine, animal husbandry and nutrition, and chemical plant protection;

and these compounds may have potential adverse effects on the environment. Besides the active ingredients (AIs), the registered formulations may contain various additives (e.g., surfactants), and in the aspects of the required main biological effect of the pharmaceutical or pesticide, these additives have long been considered as inactive or inert components. However, possible adverse effects of veterinary drugs and plant protection products may be caused not only by the AI(s), but also by the applied additives in these formulations. Several studies proved combined additive, synergistic or antagonistic side effects between the AIs and their additives used in the formulations, additionally the significantly higher own toxicity has been verified for several additives (e.g. polyethoxylated tallow amine, POEA) [1-7].

In the last decade the occurrence of the residues of veterinary pharmaceuticals in the aquatic environment have become a matter of concern, according to their potential risks posed to non-target organisms and the potential for human exposure via the food chain and drinking water. Thus, these compounds represent significant environmental loads due to the appearance of their metabolites and decomposition products in environmental matrices (e.g., soil, sediment, surface water) and even in wastewater [8-9]. According to Iglesias *et al.*, the most frequently detected pharmaceuticals in surface water were decoquinatone, sulphamethazine (SMZ), sulphamethoxypyridazine and trimethoprim (TRI) [10].

Advanced oxidation processes (AOPs) due to their high efficiency in the removal of persistent organic pollutants and pharmaceutical residues are in the focus of interest, as complementary or alternative methods to traditional wastewater treatment [11-12]. During AOP treatment of wastewater, hydroxyl ( $\cdot\text{OH}$ ) or sulphate ( $\cdot\text{SO}_4^{2-}$ ) radicals are generated in sufficient quantity to remove organic materials, organic and inorganic contaminants, or to increase the biodegradability of wastewater prior to biological treatment [13]. Application of AOP resulted in the appearance of  $\text{H}_2\text{O}_2$  in the treated solutions, when using particular methods (e.g.,  $\text{O}_3/\text{H}_2\text{O}_2$ ) or it forms in radical reactions (e.g., ionising radiation) [14], and can modify the inhibitory effects on living organisms [12,15].

The aim of this study was to investigate and compare the individual acute toxic effects of various veterinary AIs (e.g., sulphonamides and TRI) and veterinary formulations (e.g., SUMETROLIM, COTRIM-E) as a combination of AIs and additives on *Daphnia magna* immobilisation. Additionally, the effects of AOP and the appearance of  $\text{H}_2\text{O}_2$  on the toxicity of sulphamethoxazole (SMX) were investigated and compared on various test organisms (*Vibrio fischeri*, *Pseudokirchneriella subcapitata* and *D. magna*) using SMX solutions oxidised during gamma irradiation.

## Experimental

### *Determination of acute toxic effects of AIs and formulations used in veterinary medicine*

To assess the individual toxic effects of veterinary AIs, acute immobilisation tests were conducted on *D. magna* according to the OECD Test No. 202 guideline [16] using solutions of sulphonamides SMX, SMZ and sulphaguanidine (SGD) and TRI. Determination of acute toxic effects of veterinary drugs, as a combination of the AIs and additives, was performed on the basis of the same guideline. Both of the investigated veterinary medicines (SUMETROLIM and COTRIM-E) contain SMX and TRI as AIs: SUMETROLIM contains 400 mg of SMX and 80 mg TRI per tablet, while COTRIM-E contains 480 mg of co-trimoxazole in 5 ml (480 mg of co-trimoxazole consists of 400 mg of SMX and 80 mg of TRI). *D. magna* juveniles used for testing were less than 24 hrs and exposed to the test substances for 48 hrs.

Aerated reconstituted ISO test water was applied during the assays with known concentrations of the AIs and formulations. The pH value of the solutions remained between

the acceptable range of 6–9 during the experiments. The temperature was  $20\pm 2^\circ\text{C}$ , with 16-hr light and 8-hr dark photoperiods. In each test five concentrations of the investigated substance and an untreated control were used in four replicates at each level. Tests were performed in triplicates for each compound individually and in formulation. Immobilisation rates were recorded upon 24 and 48 hrs of exposure, and were compared to the untreated control values. The criteria of the test were verified.  $\text{EC}_{50}$  values were determined by statistics analysis at 48 hrs, calculated by statistical software ToxRat®. A theoretical value of the 48-hr  $\text{EC}_{50}$  value for SUMETROLIM was calculated using the nominal inhibitory concentrations of both AIs ( $\text{EC}_{50}[\text{AI}]$ ) as well.

#### *Determination of the effects of AOP treatment and the appearance of $\text{H}_2\text{O}_2$*

An aqueous solution of SMX was prepared at a concentration of  $0.1 \text{ mmol dm}^{-3}$ . The initial concentration was controlled by liquid chromatography tandem mass spectrometry (LC-MS/MS). Gradient type elution and positive ionisation mode was applied with electrospray ionisation. AOP was carried out at room temperature by a  $^{60}\text{Co}$  panoramic type  $\gamma$ -irradiation facility. Prior to the irradiation, unbuffered samples ( $1 \text{ dm}^{-3}$ , in amber glass bottles) were air saturated and were permanently aerated during the procedure. The solutions irradiated at 1 kGy absorbed dose contained hydroxylated products, but initial molecules were also present in low amounts [17-18]. Prolonged irradiation with 2.5 kGy led to decomposition of all initial molecules and resulted in the appearance of low molecular mass acids [18]. During the irradiation,  $\text{H}_2\text{O}_2$  was formed in radical reactions, and in purified water matrix it proved to be persistent. In order to make reliable ecotoxicity assays after irradiation,  $\text{H}_2\text{O}_2$  content was removed/reduced by catalytic decomposition with  $\text{MnO}_2$ .  $\text{H}_2\text{O}_2$  concentration was measured with the Merck  $\text{H}_2\text{O}_2$  test kit by spectrophotometric measurement of the absorbance at 454.5 nm of yellow or orange complexes formed.

To evaluate the effects of  $\text{H}_2\text{O}_2$  on *D. magna*, acute immobilisation tests were executed on the basis of the corresponding OECD guideline. The growth inhibition on freshwater unicellular microalgae *P. subcapitata* was investigated after 72 hrs of exposure according OECD Test No. 201 [19]. Reduction of cell growth was evaluated by measuring optical density changes at 750 nm by a UV/Vis spectrophotometer (JASCO 550). The samples were constantly shaken (100 rpm) and illuminated continuously (8600-8800 lux). Acute toxicity of SMX and  $\text{H}_2\text{O}_2$  on *V. fischeri* a widely used bioluminescent bacterium, was determined by Microtox® tests performed on the basis of the adequate protocol approved by US-EPA [20]. The inhibition of natural light emission was determined compared to a non-toxic control. The detected decrease in luminescence and the increase in toxicity are proportional. Inhibition was evaluated after 30 min of exposure at pH  $7\pm 0.2$ . The tests were performed in triplicates by using two parallels.

#### **Results and discussion**

On the basis of our acute toxicity testing on *D. magna* the most toxic AI was SGD ( $\text{EC}_{50} = 1.79\pm 0.34 \text{ mg L}^{-1}$ ), SMZ was less toxic ( $\text{EC}_{50} = 38.07\pm 9.52 \text{ mg L}^{-1}$ ), while the least toxic veterinary AIs were the SMX and trimethoprim (TRI) with evaluated  $\text{EC}_{50}$  values of  $98.06\pm 58.67$  and  $93.05\pm 33.2 \text{ mg L}^{-1}$ , respectively. Significant differences were observed in the toxicity of the investigated veterinary drugs containing SMX and TRI. SUMETROLIM was more toxic on *D. magna* ( $\text{EC}_{50} = 106.17\pm 54.86 \text{ mg L}^{-1}$ ) compared to COTRIUM-E (its concentration of  $250 \text{ mg L}^{-1}$  resulted in 15% immobilisation). The combined toxicity of SMX and TRI was higher when the two AIs were investigated together in equivalent concentrations, than in the

formulated product SUMETROLIM. The  $EC_{50}[AI]$  values of SMX and TRI corrected to SUMETROLIM were  $71.13 \pm 36.75$  and  $13.80 \pm 7.13$  mg L<sup>-1</sup>, respectively.

On *V. fischeri* the untreated SMX solution showed  $5 \pm 1\%$  inhibition, while  $30 \pm 2\%$  inhibition was observed in both irradiated solutions at 1 kGy and 2.5 kGy. The toxicity of SMX solutions increased in function of the quantity of absorbed dose, and was significantly higher in the presence of H<sub>2</sub>O<sub>2</sub>. It can be concluded that the presence of H<sub>2</sub>O<sub>2</sub> due to AOP has a significant impact on the exposure of the test organisms and on the results. To investigate the impact of H<sub>2</sub>O<sub>2</sub> alone on the test organisms (*D. magna*, *P. subcapitata* and *V. fischeri*), experiments have been conducted using a dilution series of H<sub>2</sub>O<sub>2</sub> aqueous solutions up to 0.5 mmol dm<sup>-3</sup>. Remarkably high inhibition was observed at the concentration of 0.5 mmol dm<sup>-3</sup> H<sub>2</sub>O<sub>2</sub> on all applied test organisms (Figure 1), resulting in  $100 \pm 0\%$ ,  $96 \pm 1\%$  and  $72 \pm 5\%$  inhibition on *D. magna*, *P. subcapitata* and *V. fischeri*, respectively. Therefore, at this concentration the presence of H<sub>2</sub>O<sub>2</sub> hinders interpretation of results targeting toxicity of products formed during the treatment. With the reduction of H<sub>2</sub>O<sub>2</sub> concentrations, decreased inhibition was observed on *V. fischeri* and *P. subcapitata*. A linear correlation was detected between the inhibition and H<sub>2</sub>O<sub>2</sub> concentrations. The inhibitory effects of H<sub>2</sub>O<sub>2</sub> (below 0.05 mmol dm<sup>-3</sup> concentration) on *V. fischeri* and *P. subcapitata* were regarded as acceptable, i.e.  $2 \pm 0\%$  and  $14 \pm 6\%$ , respectively. *D. magna* showed a different behaviour, where the concentration-response curve was sigmoidal. The toxicity was not modified with the reduction of H<sub>2</sub>O<sub>2</sub> concentration from 0.5 to 0.1 mmol dm<sup>-3</sup>. The reduction of H<sub>2</sub>O<sub>2</sub> concentration resulted in a decrease of immobilisation from  $90 \pm 9\%$  to  $24 \pm 9\%$  (Figure 1). The acceptable susceptibility of these organisms was detected when the level of H<sub>2</sub>O<sub>2</sub> was decreased to 0.01 mmol dm<sup>-3</sup> (resulting in  $6 \pm 8\%$  immobilisation) or below. The evaluated EC<sub>50</sub> values were found to be as high as 0.349, 0.251 and 0.064 mmol dm<sup>-3</sup> for *V. fischeri*, *P. subcapitata* and *D. magna*, respectively [12].

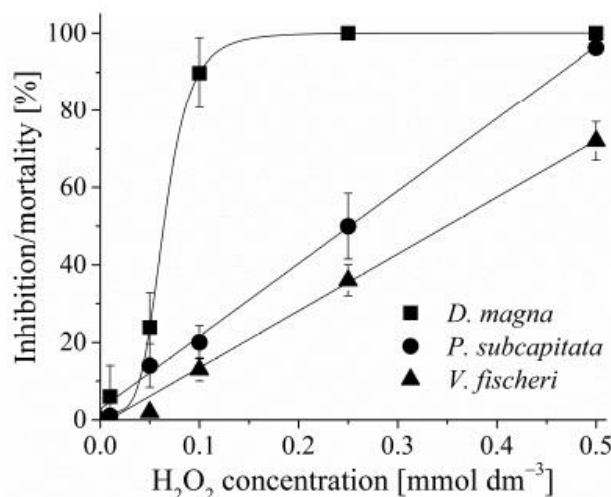


Figure 1. Concentration dependence of H<sub>2</sub>O<sub>2</sub> effects on *Daphnia magna*, *Pseudokirchneriella subcapitata* and *Vibrio fischeri* [12]

### Conclusion

On the basis of scientific data, the (eco)toxicity evaluation of surfactants and other additives is necessary for sufficient environmental risk assessment of formulations used in agriculture

including veterinary medicines, animal husbandry and plant protection. In addition, these components cannot be classified as inactive components regarding their side-effect profiles, due to their properties and their role in biological interactions. Our results emphasise the investigation and refinement of the complementary or alternative methods useable in traditional wastewater treatment, like AOP treatments. Residual H<sub>2</sub>O<sub>2</sub> in AOP may significantly modify the results of ecotoxicity assessment using living test organisms. During AOP treatments, a substantial reduction of H<sub>2</sub>O<sub>2</sub> is recommended to at least ~0.05 mmol dm<sup>-3</sup> in *V. fischeri* and *P. subcapitata* investigations due to the significant inhibition by H<sub>2</sub>O<sub>2</sub> at higher concentrations. In case of *D. magna*, complete elimination of H<sub>2</sub>O<sub>2</sub> is needed prior to tests, in order to avoid misleading results during the investigation of the effects of AOP on the toxicity of the treated solutions.

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