



## Mini Review

# Mechanism-related teratogenic, hormone modulant and other toxicological effects of veterinary and agricultural surfactants

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## Summary

Veterinary and agricultural surfactants are supposed to be inert additives, yet these substances commonly exert biological side-effects, in given cases synergistic with those of the active ingredients of these preparations. This is explicitly seen in altered toxicity of veterinary or pesticide formulations compared to their active ingredients alone. Neither the individual effects of these excipients, nor such combination effects are well-studied in toxicology, and therefore, possible toxicity consequences are occasionally not being considered at sufficient significance in the authorization, use and control of these substances. Risk assessment of these substances should cover all hazards they may represent, and corresponding levels of exposure. Surfactants used in veterinary and pesticide formulation enter the environment either by direct dispersion or by indirect release through excrement, leaching, sewage waters or sludge, and in turn, create potential exposure to a number of non-target organisms. Biochemical and (eco)toxicological hazards recently identified regarding certain agricultural surfactants include cytotoxicity (on cell lines of epithelial, neural and other tissues, as well as stem cells and tumor cells), endocrine disrupting effects, as well as aquatic ecotoxicity. This Mini Review summarizes toxicological effects identified in our studies in aquatic toxicity tests, in cell viability and cytotoxicity tests, in estrogenic activity assays, correlated with biochemical analysis of the surfactants and their decomposition. The conclusions are hoped to facilitate environmentally precautionous revision of surfactants widely used in agriculture.

## Introduction

The world production of surfactants is nearly 15 million tons/year. Although agricultural surfactants, mostly used for the formulation of veterinary drugs and pesticides [1], they represent a significant, yet minor proportion compared to industrial surfactants: the agricultural surfactants market is expected to be worth 1.53 billion USD by 2020 [2]. This volume is sufficient to be considered as a source of substantial environmental exposure, as these substances are practically fully released into the environment. Veterinary surfactants include feed additives, e.g. dioctyl sodium sulfosuccinate, as well as cleansers, antiseptics and disinfectants. Main representatives are cationic tensides, benzalkonium chlorides and benzoxonium chloride, with antibacterial and antiviral activity (the use of which being limited by the reported emergence of bacterial resistance), but anionic, non-ionic surfactants and biotensides are also found in this group [3]. Adjuvants for pesticide formulations constitute a broad range of substances with solubilizers and adsorption enhancers as the major types. Non-ionic surfactants, fatty alcohol/amine ethoxylates (AEOs/ANEOS) are typical examples of pesticide adjuvants, but anionic tensides e.g., alkylpolyoxyethylene ether phosphates, alkyl phosphate esters, alkyl sulfosuccinate, alkyl polyglucoside sulfosuccinate and alkyl polyglucoside citrate salts, as well as sodium alkylbenzene sulfonates are also widely applied in formulated products or in tank mixtures.

Adjuvant surfactants are used primarily to enhance the biological effect of the active ingredient, therefore, directly affect the efficiency of the formulations. Surfactants may enhance the efficiency of the formulation by modifying the physico-chemical characteristics (e.g. water solubility) or bioavailability of the active ingredients [4,5]. Surfactants may also be applied in veterinary drugs as micellar solubilizers or in feed additives and in drinking water as stabilizers. Active ingredients in both veterinary drug and pesticide formulations are responsible for the main (therapeutic or plant protection) effects of the preparation. Co-formulants, e.g. surfactants cannot exert such main effects *per definitionem*, as otherwise they would be also considered as active ingredients. Due to this definition, formulating surfactants have been considered as 'inert' ingredients. Concluding from the above definition, co-formulants may not exert the main effects of the formulation, however, such inertness cannot warrant against unintended detrimental side-effects. The significance of potential effects of unidentified and assumedly inert pesticide ingredients on human and environmental health has been emphasized earlier [6,7]. The more complex the interaction of those substances with the potentially exposed organisms is, the broader the possibility of the occurrence of such side-effects becomes.

Both veterinary and pesticide formulation surfactants enter the environment either by direct dispersion or by indirect release through excrement, leaching, sewage waters or sludge: cumulated AEO concentrations in ground water were found to be 710 ng/l (61-189 ng/l for 6 AEO homologs) [8,9]. Little is known about the environmental fate of adjuvants after application on agricultural land, although the adverse effects are numerous. Nonylphenol ethoxylates (NPES) have been found to exert mild to medium estrogenic function [10]. Cocamide diethanolamine (coconut oil DEA) is an IARC "2B" carcinogen [11], which identifies this chemical as possibly carcinogenic to humans.

Individual toxicity is usually well-revealed in toxicology studies, while assessing the effects of substance combinations is severely problematic, as antagonizing, additive and synergistic effects often result in altered toxicological profiles. Numerous cases are known, when an active ingredient and an adjuvant do not show significant side-effects themselves, but their finished preparation, on the contrary, is of pronounced effect. Such role of production contamination is often identified (e.g. the case of Agent Orange in the Vietnam War). While information on the active ingredients of given preparations is readily available, the formulation technology of these preparations is concealed by manufacturers' copyright protection. A common feature of these protocols that formulation components are declared as inert.

### Chemical analysis of veterinary and agricultural surfactants

To determine residue levels and to assess toxicity, proper methods of chemical analysis need to be developed. The often complex and not exactly defined composition of surfactant mixtures, as well as the lack of reliable data and reference materials renders such development rather difficult. The broad variety of the chemical composition and wide polarity characteristics of these substances also presents difficulties in environmental analysis. Thus, conventional analytical methods are usually insufficient to detect surfactants from different classes. Analytical methods based on gas chromatography (GC), often upon chemical derivatization, are suitable to detect surfactant compounds, for example, alcohol sulfates have to be hydrolyzed to alcohols and silylated for GC analysis [12]. Aromatic surfactants are determined by high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection, but also only after chemical derivatization [13,3]. Consequently, mainly liquid chromatography coupled to mass spectrometry (LC-MS) is being applied for the analysis of surfactants [14], and the first multiple-class analytical method for surfactants, suitable for environmental monitoring purposes, was published only recently [15]. An additional difficulty is that the oligomer distribution in given surfactants e.g., polyethoxylated tallowamine (POEA), may vary among formulations [16], and the environmental fate of the surfactant may also affect homolog distribution pattern [17].

### Toxic effects of surfactants

Surfactants used in veterinary drugs or pesticide formulations may have adverse effects on the environment and on non-target organisms. The cytotoxicity order of surfactants investigated on rabbit corneal epithelial cells was found to be cationic > anionic = amphoteric > non-ionic [18]. The simultaneous application and presence of non-ionic amine oxide-based surfactants and anionic surfactants in formulations has been proven to result in synergistic effects between the surfactants [19,20]. Surfactants may influence the embryonic development and hormonal balance of vertebrates, mainly in aquatic habitats, and genotoxic effects have been indicated for several types of surfactants [21-25]. Non-ionic detergents (decyl polyglycoside) exerted higher toxicity on algae, than anionic (e.g., sodium lauryl ether sulfate and ALS) or amphoteric (alkylamidopropyl betaine and alkylamidoethyl-N-hydroxyethyl glycine) ones [26]. Nonylphenol and octylphenol as biodegradation products of alkylphenol ethoxylates exert toxicity on freshwater and marine fish [27] and induce estrogenic responses [28,29]. Moreover, the individual toxicity of POEA was verified as well [30].

### Differential toxicity of formulated veterinary and pesticide preparations compared to their active ingredients

Interactions may occur between active ingredients and additives used in formulated veterinary drugs or pesticides [31,32]. Combined effects of active ingredients and surfactants have been confirmed in veterinary medicine, e.g. antagonistic effects between various bacteriostatic and bactericidal compounds, synergistic effects between antiseptic ionic and non-ionic tensides [33,34] and hexachlorophene [35]. Moreover, the dissociation,  $\alpha$ -chymotryptic degradation and enteral absorption of insulin hexamers are influenced by combination of sodium dodecyl sulfate and the cationic cetyl trimethyl ammonium bromide surfactants in pharmaceuticals [36].

Veterinary drugs and their components are subject to stricter authorization regulations and more detailed effect assessment than plant protection products, moreover, their chemical composition is better defined for the public. Our aquatic toxicity studies carried out with a veterinary drug Sumetrolim on the great water flea (*Daphnia magna* Straus) [37,38,39] indicated that the toxicity of the preparation ( $LC_{50}$ =106.2 mg/L) was 25% higher than it would be expected from the individual toxicities of the active ingredients sulfamethoxazol and trimethoprim, indicating a synergistic effect between the active ingredients or between formulating additives and the active ingredients (Takács et al., unpublished). Studies by our team and other research groups have also justified the concerns raised earlier regarding potential side-effects on human and environmental health by assumedly inert ingredients in pesticide formulations [6]. Several pesticide formulations were proven to exert higher cytotoxicity on human cell lines than their active ingredients alone [40]. Recently, we have evidenced approximately 50-fold higher toxicity of a formulated neonicotinoid insecticide product (Apache 50 WG) than its active ingredient (clothianidin) in acute immobilization tests on *D. magna* [3]. Interestingly, formulated insecticides containing other neonicotinoid active ingredients (thiacloprid, thiamethoxam) appeared to be 2-3 times less toxic than these active ingredients. These results indicated possible synergistic/antagonistic interactions of the formulating agents with the active ingredients.

A particularly exposed issue has been the case of POEA used as a surfactant for herbicide active ingredient glyphosate. The US Environmental Protection Agency (EPA) found POEA being more toxic to fish than the active ingredient it is applied with (glyphosate) [41]. Differential toxicity of glyphosate and its POEA-formulated preparations have been evidenced [22,25,40,42,43]. Neural defects and craniofacial malformations were found in regions where corresponding herbicides were used in a survey on embryologic effects (including endogenous retinoid activity) on amphibian species [23], linked to the retinoic acid signaling pathway.

In our studies to test short-term ecotoxicity of the surfactant excipients, *in vivo* assays by OECD standards were carried out on indicator organisms, e.g. *D. magna*, unicellular green algae (*Pseudokirchneriella subcapitata*, *Desmodesmus subspicatus*, *Scenedesmus obtusiusculus*), as well as zebrafish (*Danio rerio*). The effects of the target substances were also determined on algal communities in biofilms. Extensive cytotoxicity assessment has been carried out on human and other mammalian cell lines, and effects on cell viability, apoptosis, cell cycle, and intactness of barrier functions were determined, along with studying genotoxicity, hormone modulant and gender determination.

When assessing glyphosate and its formulations, we found the co-formulant to affect the aqueous environmental fate of the active ingredient both in absence and particularly in the presence of algal biofilms [44]. In the ecotoxicity tests, the surfactants were proven to be the most toxic in all tests systems, but different surfactants showed varying toxicity, well-correlated with the toxicities of the formulated preparations. In our hands, POEA and a POEA-formulated glyphosate preparations were found equitoxic on the aquatic toxicity test on *D. magna*, 150-300-fold more toxic than the active ingredient [45]. The effect of POEA was found to be the greatest (being 2000-fold more toxic than the active ingredient in cell viability and zebrafish teratogenicity tests), the same substances showed four orders of magnitude higher cytotoxicity than glyphosate on a murine neuroectodermal stem cell-like cell line [43]. Our studies included testing several glyphosate-based herbicide preparations, along with glyphosate and several formulant surfactants in the fish embryotoxicity test (FET test) using zebrafish (*D. rerio*) embryos [46], and treated embryos were screened in addition for signs of acute toxicity, for teratogenic deformities (coagulation of fertilized eggs, lack of somite formation, lack of detachment of the tail-bud from the yolk sac, and lack of heartbeat). The surfactants tested (POEA, alkyl polyglucoside sulfosuccinate and citrate salts) were found to be the most toxic ( $LC_{50}$ =3.9-5.0 mg/L), while various formulated herbicide preparations and glyphosate were 6-50-fold and 2200-fold less toxic, respectively. Sublethal deformities and edemas, as well as signs of teratogenicity were seen near or above the  $LC_{50}$  values. To test whether teratogenicity was being linked to the retinoic acid pathway, the levels of all-trans-retinoic acid, 13-cis-retinoic acid and retinol were determined in zebrafish embryos and throughout development into adulthood (24h, 72h, 1-, 3- and 5-week old). The limits of detection in the applied HPLC-UV method were 0.5 ng, 1.0 ng and 0.2 ng for all-trans-retinoic acid, 13-cis-retinoic acid and retinol, respectively. All-trans-retinoic acid was detected in all samples, but its level does not change significantly during the development. The levels of trans-retinol increased during this period, while 13-cis-retinoic acid was not detected [47].

Our studies detected cytotoxic effects on human HEK293 and murine NE-4C cells by glyphosate, its formulated herbicide and adjuvant POEA. The formulated pesticide and POEA were found to be equitoxic at short exposures ( $LC_{50}$ =10-15 ng/mL in 6 hr), while glyphosate occurred to be of 500-750-fold less toxic. POEA was found cytotoxic above 1 ng/mL concentration on human cell lines after 2 to 24 hrs of exposure [47]. Cytotoxicity was detected not only in colorimetric cell viability tests, but also by the innovative visualization method, holographic microscopy [43,48]. Similar cytotoxicity was observed through reduced proliferation and lower tumor-associated glycosphingolipid expression (particularly of GD3 gangliosides, highly tumor-associated antigens) seen in MDA MD-231 and MCF7 breast cancer cell lines [49]. Moreover, glyphosate-based preparations containing several surfactants besides POEA and the surfactants themselves were found to exert cytotoxicity orders of magnitude higher than the active ingredient, and were found to inhibit aromatase (a key enzyme in estrogen biosynthesis) indicating endocrine disrupting effects as well [50]. Among the surfactants studied, those with the least level of toxicity were found to be alkylpolyglycosides. POEA modifies cell permeability and may amplify the effect of biologically active substances through cell apoptosis and necrosis. As indicated, several surfactants can influence animal health, embryonic development and further hormonal balance in vertebrates, especially in aquatic environments.

## Future directions and recommendations

The toxic effects (acute toxicity on indicator species (*D. magna*, *D. rerio*), cytotoxicity on various cell lines of animal and human origin, endocrine disruptive characteristics, genotoxicity and teratogenicity) identified in the scientific literature and in this paper summarizing the results of a targeted research project, regarding non-target toxicity of active ingredients and formulating surfactants used in veterinary drugs and pesticide products, call for continuing investigations and characterization of toxic interactions between these active ingredients and surfactants, on the one hand; and lead to certain recommendations, on the other hand. (1) The concept that additives used in veterinary drug and pesticide formulations are inert ingredients needs to be abandoned. Additives may not exert activity related to the main effect of the active ingredients, but can be causative agents for a wide range of unintended adverse side-effects, just like the active ingredient. (2) Stricter toxicological and ecotoxicological assessment of agricultural surfactants used in veterinary drugs and pesticide products is needed. As these additives are applied on livestock and released into the environment together with the active ingredient(s), they cause similar exposures, and the extent of the anticipated exposures and hazards related need to be evaluated and regulated as strictly as required for the active ingredients. (3) A well-defined harmonization in authorization and regulatory policies between veterinary drugs and pesticide products is needed. Toxicity characteristics of the formulated pesticide products (and not the active ingredients and additives separately) need to be considered in decision-making, similarly as required for veterinary drugs. This is of particular concern for tank mixtures. (4) More detailed labeling information requirements need to be set for compositional information of veterinary drugs and pesticide products requesting to reveal the exact identity and content of the formulating surfactants they contain.

## Conclusion

It has been proven in several instances on the excipients of formulated veterinary drugs and pesticide products, that the additives used in them (considered inert with respect of the main (therapeutic) effect of the products) may exert side-effects of their own, and therefore, their usage needs to be more strictly regulated. Combination effects are not well studied in toxicology. Scientific evidence on the properties of certain veterinary and pesticide formulants, particularly tallow derivatives, and on their role in various biological interactions indicate that these substances cannot be considered as unequivocally inactive ingredients by ecotoxicological and toxicological aspects in the toxicological and environmental risk assessment of veterinary drugs and pesticide formulations. To assure that no unexpected detrimental side-effects are exerted by these excipients, their full toxicological assessment and evaluation is justified in many cases to assure proper and effective environmental and food/feed safety of formulations used in veterinary and agricultural practice.

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