Chapter 3

What Do We Know About the Chronic and Mixture Toxicity of the Residues of Sulfonamides in the Environment?

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Additional information is available at the end of the chapter

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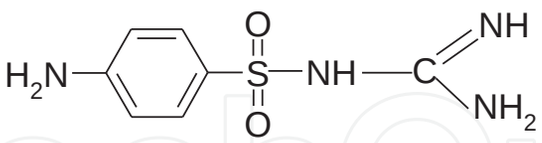
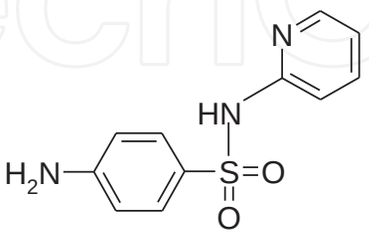
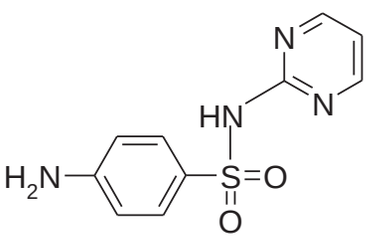
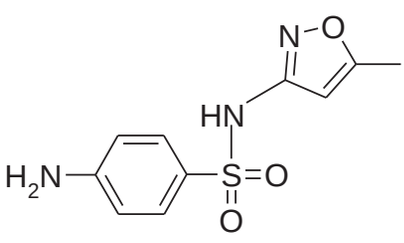
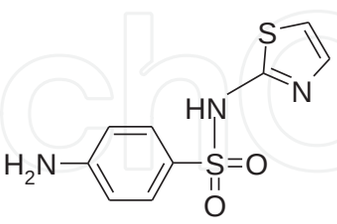
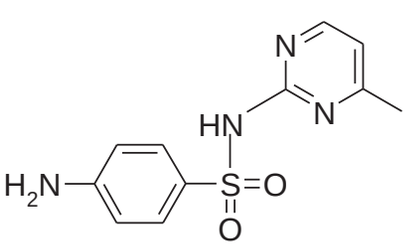
1. Introduction

Thanks to their low cost and their broad spectrum of activity in preventing or treating bacterial infections, sulfonamides (SAs) are one of the oldest groups of veterinary chemotherapeutics, having been used for more than fifty years. To a lesser extent they are also applied in human medicine. After tetracyclines, they are the most commonly consumed veterinary antibiotics in the European Union. As these compounds are not completely metabolized, a high proportion of them are excreted unchanged in feces and urine. Therefore, both the unmetabolized antibiotics as well as their metabolites are released either directly to the environment in aquacultures and by grazing animals or indirectly during the application of manure or slurry [1-3].

Physico-chemical properties and chemical structures of selected SAs are presented in Table 1. They are fairly water-soluble polar compounds, the ionization of which depends on the matrix pH. All the sulfonamides, apart from sulfaguanidine, are compounds with two basic and one acidic functional group. The basic functional groups are the amine group of aniline (all the SAs) and the respective heterocyclic base, specific to each SA. The acidic functional group in the SAs is the sulfonamide group. With such an SA structure, these compounds may be described by the pK_{a1}, pK_{a2} and pK_{a3} values corresponding to the double protonated, once protonated and neutral forms of SA (Table 1) [3-7].

Substance [CAS] Abbreviation	**Chemical structure**	**Selected physico-chemical properties**
Sulfaguanidine [57-67-0] SGD		M = 214.2 g mol^{-1} pK_{a2} = 2.8 pK_{a3} = 12.0 logP = -1.22
Sulfapyridine [144-83-2] SPY		M = 249.2 g mol^{-1} pK_{a2} = 2.37 pK_{a3} = 7.48 logP = 0.03
Sulfadiazine [68-35-9] SDZ		M = 250.3 g mol^{-1} pK_{a2} = 1.98 pK_{a3} = 6.01 logP = -0.09
Sulfamethoxazole [723-46-6] SMX		M = 253.3 g mol-1 pK_{a2} = 1.81 pK_{a3} = 5.46 logP = 0.89
Sulfathiazole [72-14-0] STZ		M = 255.3 g mol^{-1} pK_{a2} = 2.06 pK_{a3}= 7.07 logP = -0.04
Sulfamerazine [127-79-7] SMR		M = 264.3 g mol^{-1} pK_{a2} = 2.16 pK_{a3} = 6.80 logP = 0.11

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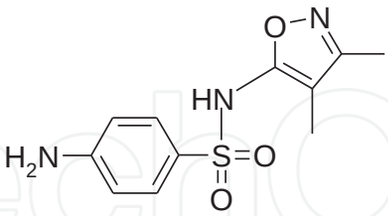
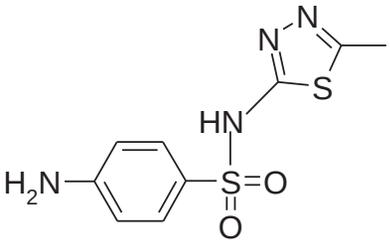
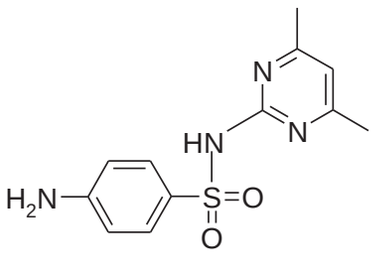
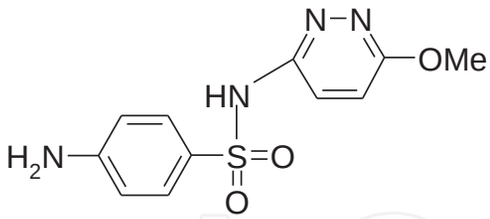
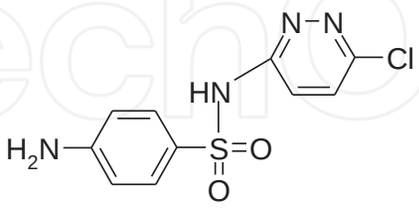
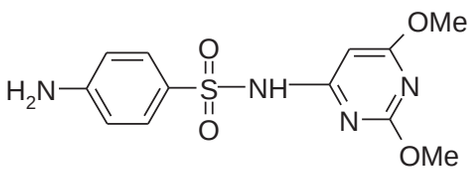
Substance [CAS] Abbreviation	**Chemical structure**	**Selected physico-chemical properties**
Sulfisoxazole [127-69-5] SSX		M = 267.3 g mol^{-1} pK_{a2} = 2.15 pK_{a3}= 5.00 logP = 1.01
Sulfamethiazole [144-82-1] SMTZ		M = 270.3 g mol^{-1} pK_{a2} = 2.24 pK_{a3} = 5.30 logP = 0.47
Sulfadimidine (sulfamethazine) [57-68-1] SDMD (SMZ)		M = 278.3 g mol^{-1} pK_{a2} = 2.46 pK_{a3} = 7.45 logP = 0.27
Sulfamethoxypyridazine [80-35-3] SMP		M = 280.3 g mol^{-1} pK_{a2} = 2.20 pK_{a3} = 7.20 logP = 0.32
Sulfachloropyridazine [80-32-0] SCP		M = 284.7 g mol^{-1} pK_{a2} = 1.72 pK_{a3} = 6.39 logP = 0.71
Sulfadimethoxine [122-11-2] SDM		M = 310.3 g mol^{-1} pK_{a2} = 2.5 pK_{a3} = 6.0 logP = 1.63

Table 1. Structures and physico-chemical properties of selected sulfonamides (according to [1,6-13])

Due to their properties, after disposal in soils, these compounds may enter surface run-off or be leached into the groundwater. Moreover, they are also quite persistent, non-biodegradable and hydrolytically stable, which explains why in the last ten years they have been regularly detected not only in aquatic but also in terrestrial environments [1-3,7,14]. Although SAs concentrations in environmental samples are quite low (at the μg L^{-1} or ng L^{-1} level), they are continuously being released [3,15]. Therefore, the kind of exposure organisms may be subjected to will resemble that of traditional pollutants (e.g. pesticides, detergents), even those of limited persistence. Consequently, SAs as well as other pharmaceuticals may be considered pseudo-persistent.

SAs are designed to target specific metabolic pathways (they competitively inhibit the conversion of *p*-aminobenzoic acid, PABA) by inhibiting the biosynthetic pathway of folate (an essential molecule required by all living organisms), so they not only affect bacteria (target organisms) but can also have unknown effects on environmentally relevant non-target organisms, such as unicellular algae, invertebrates, fish and plants [16-18]. Belonging to different trophic levels, these taxonomic groups may be exposed to by SAs to various extents [15-16,19-20].

However, knowledge of the potential effects of SAs on the environment is very limited. Recently, a few review papers have been published that summarize the available ecotoxicity data of pharmaceuticals, including some sulfonamides [16-17,19-21]. Such data as are available on the potential effects of pharmaceuticals in the environment appear to indicate a possible negative impact on different ecosystems and imply a threat to public health. However, if we look just at the sulfonamides, most current studies have investigated acute effects mainly of single compounds and mostly with reference to sulfamethoxazole (SMX), one of the most common SAs, used in both veterinary and human medicine [16-17,20]. Available information on the ecotoxicity of selected sulfonamides has been review and is presented in Table 2.

Substance	Bacteria *Vibrio fischeri*	Green algae / Cyanobacteria / Diatom*	Plants**	Invertebrates***		Vertebrates****
SGD	$>50_{(30\ min)}$	$43.56_{(96h,\ P.\ subcapitata)}$	$30.30_{(7d,\ L.gibba)}$	$0.87_{(48h,\ D.\ magna)}$		
		$3.40_{(96h,\ S.\ dimorphus)}$	$0.22_{(7d,\ L.\ minor)}$			
		$16.59_{(96h,\ S.\ leopoliensis)}$				
		$3.42_{(24h,\ S.vacuolatus)}$				
SPY	$>50_{(30\ min)}$	$5.28_{(24h,\ S.vacuolatus)}$	$0.46_{(7d,\ L.\ minor)}$			
SDZ	$>25_{(30\ min)}$	$7.80_{(72h,\ P.\ subcapitata)}$	$0.07_{(7d,\ L.\ minor)}$	$221_{(48h,\ D.\ magna)}$		
		$2.19_{(72h,\ P.\ subcapitata)}$		$13.7_{(21d,\ D.\ magna)}$		
		$0.135_{(72h,\ M.\ aeruginosa)}$		$212_{(48h,\ D.\ magna)}$		
		$2.22_{(24h,\ S.vacuolatus)}$				
SMX	$23.3_{(30\ min)}$	$1.53_{(72h,\ P.\ subcapitata)}$	$0.081_{(7d,\ L.gibba)}$	$189.2_{(48h,\ D.magna)}$	$123.1_{(48h,\ D.magna)}$	$>750_{(48h,\ O.\ latipes)}$ [a]
	$>84_{(30\ min)}$	$0.15_{(96h,\ P.\ subcapitata)}$	$0.132_{(7d,\ L.gibba)}$	$177.3_{(96h,\ D.magna)}$	$205.1_{(48h,\ D.magna)}$	$562.5_{(96h,\ O.\ latipes)}$ [a]
	$78.1_{(15\ min)}$	$0.52_{(72h,\ P.\ subcapitata)}$	$0.0627_{(14d,\ D.carota)}$	$25.2_{(24h,\ D.\ magna)}$	$70.4_{(48h,\ M.macrocopa)}$	$27.36_{(24h,\ O.\ myskiss)}$
	$74.2_{(5\ min)}$	$2.4_{(96h,\ C.\ meneghiniana)}$	$0.0612_{(21d,\ D.carota)}$	$15.51_{(48h,\ C.\ dubia)}$	$84.9_{(24h,\ M.macrocopa)}$	
	$>100_{(30\ min)}$	$0.0268_{(96h,\ S.\ leopoliensis)}$	$0.0454_{(28d,\ D.carota)}$	$0.21_{(7d,\ C.\ dubia)}$	$9.63_{(48h,\ B.calyciflorus)}$	

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Substance	Bacteria *Vibrio fischeri*	Green algae / Cyanobacteria/ Diatom*	Plants**	Invertebrates***	Vertebrates****
		$1.54_{(24h, S.vacuolatus)}$	$0.21_{(7d, L. minor)}$	$100_{(48h, C. dubia)}$ $35.36_{(24h, T.platyurus)}$ [a]	
STZ	$>1000_{(15min)}$	$13.10_{(24h, S.vacuolatus)}$	$3.552_{(7d, L.gibba)}$	$149.3/85.4_{(48h/96h, D. magna)}$	$>500_{(48h, O. latipes)}$ [a]
	$>50_{(30 min)}$		$4.89_{(7d, L. minor)}$	$616.7_{(24h, D. magna)}$	$>500_{(96h, O. latipes)}$ [a]
				$391/430_{(48h/24h, M. macrocopa)}$	$>100_{(48h, O. myskiss)}$ [a]
				$135.7/78.9_{(48h/96h, D. magna)}$	
SMR	$>50_{(30 min)}$	$11.90_{(24h, S.vacuolatus)}$	$0.68_{(7d, L. minor)}$		
SSX	$>50_{(30 min)}$	$18.98_{(24h, S.vacuolatus)}$	$0.62_{(7d, L. minor)}$		
SMTZ	$>100_{(30 min)}$	$24.94_{(24h, S.vacuolatus)}$	$2.54_{(7d, L. minor)}$		
SDMD	$344.7_{(15 min)}$	$19.52_{(24h, S.vacuolatus)}$	$1.277_{(7d, L. gibba)}$	$174.4/158.8_{(48h/96h, D. magna)}$	$>500_{(48h, O. latipes)}$ [a]
	$>100_{(30 min)}$		$1.74_{(7d, L. minor)}$	$215.9/506.3_{(48h/24h, D. magna)}$	$>500_{(96h, O. latipes)}$ [a]
				$111/311_{(48h/24h, M. macrocopa)}$	
				$185.3/147.5_{(48h/96h, D. magna)}$	
SMP	$>100_{(30 min)}$	$3.82_{(24h, S.vacuolatus)}$	$1.51_{(7d, L. minor)}$		
SCP	$26.4_{(15 min)}$	$32.25_{(24h, S.vacuolatus)}$	$2.33_{(7d, L. minor)}$	$375.3/233.5_{(48h/96h, D. magna)}$	$589.3_{(48h, O. latipes)}$ [a]
	$>50_{(30 min)}$		$2.48_{(7d, L. minor)}$		$535.7_{(96h, O. latipes)}$ [a]
SDM	$>500_{(15 min)}$	$2.30_{(72h, P. subcapitata)}$	$0.445_{(7d, L.gibba)}$	$248.0/204.5_{(48h/96h, D. magna)}$	$>100_{(48h, O. latipes)}$ [a]
	$>500_{(5 min)}$	$11.2_{(72h, C. vulgaris)}$	$0.248_{(7d, L.gibba)}$	$270/639.8_{(48h/24h, D. magna)}$	$>100_{(96h O. latipes)}$ [a]
	$>50_{(30 min)}$	$9.85_{(24h, S.vacuolatus)}$	$0.02_{(7d, L. minor)}$	$184/297_{(48h/24h, M. macrocopa)}$	
SQO [b]		$0.25_{(96h, P. subcapitata)}$	$13.55_{(7d, L.gibba)}$	$3.47_{(48h, D. magna)}$	
		$0.45_{(96h, S. dimorphus)}$	$2.33_{(7d, L. minor)}$		
		$2.83_{(96h, S. leopoliensis)}$			

* green algae: *Pseudokirchneriella subcapitata* (previously *Scenedesmus capricornutum*), *Scenedesmus dimorphus, Chlorella vulgaris*; cyanobacteria *Synechococcus leopoliensis, Microcystis aeruginosa*; diatom *Cyclotella meneghiniana*;

** duckweed *Lemna gibba*, *Lemna minor*, carrot *Daucus carota*;

*** crustacean: *Moina macrocopa, Clathrina dubia, Thamnocephalus platyurus, Daphnia magna*; rotifer: *Brachionus calyciflorus*;

**** fish: *Oryzias latipes*, rainbow trout *Onchorhynchus mykiss*;

Table 2. Summary of the ecotoxicological risk (described by EC_{50} or LC_{50} [a] in mg L^{-1}) estimated for different sulfonamides (data obtained from [16,19-20,22-33]); [b] sulfaquinoxaline

This demonstrates the lack of data relating to the long-term exposure of non-target organisms, and especially how continuous exposure for several generations may affect a whole population. Moreover, as these compounds occur in natural media not as a single, isolated drug but usually together with other compounds of the same family or the same type, accumulated concentrations or synergistic-antagonistic effects can be also observed. The simultaneous presence of several pharmaceuticals in the environment may result in a higher level of toxicity towards non-target organisms than that predicted for individual active substances.

Therefore, the main aim of this chapter was to review the existing knowledge on the chronic and mixture toxicity of the residues of sulfonamides in the environment, since it has not been done yet. This will be achieved by: (1) presenting current approaches for Environmental Risk Assessment (ERA) for pharmaceuticals with respect to the evaluation of chronic and mixture toxicity of these compounds; (2) introducing the reader to basic concepts of chemical mixture toxicology; and finally (3) by discussing detailed available information on chronic and mixture toxicity of the residues of sulfonamides in the environment.

2. Environmental risk assessment of pharmaceuticals vs. chronic and mixture toxicity of pharmaceuticals

The approaches currently being used to assess the potential environmental effects of human and veterinary drugs in the U.S. and in the European Union are in some respects dissimilar [34-39]. The Environmental Risk Assessment (ERA) process usually starts with an initial exposure assessment (Phase I). But with some exceptions, a fate and effects analysis (Phase II) is only required when exposure-based threshold values, the so-called action limits, are exceeded in different environmental compartments. Thus risk assessment, described by Risk Quotient (RQ), is performed by the calculation the ratio of the predicted (or measured) environmental concentration (PEC or MEC respectively) and predicted biological non-effective concentrations (PNEC) on non-target organisms. If RQ is less than one it indicates that no further testing is recommended. Calculations of environmental concentrations rely e.g. on information on treatment dosage and intensity along with default values for standard husbandry practices, and are based on a total residue approach reflecting worst-case assumptions. For example, the recently introduced European guidance on assessing the risks of human drugs excludes the testing of pharmaceuticals whose $PEC_{surface\ water}$ is below an action limit of 0.01 μg L^{-1}; in the U.S. this threshold value is 0.1 μg L^{-1}. Moreover, there are two different action limits for veterinary pharmaceuticals, one each for the terrestrial and the aquatic compartments. No fate and effect analysis is required for veterinary pharmaceuticals used to treat animals if the PEC_{soil} is < 100 μg kg^{-1} dry weight of soil. However, a Phase II assessment is not required for veterinary medicines used in an aquaculture facility if the estimated concentration of the compound is < 1 μg L^{-1} [40-41]. If the $PEC_{surface\ water}$ of a pharmaceutical is above the action limit, effects on algae, crustaceans and fish are investigated. However, if PEC_{soil} is higher than the action limit, then Phase II, divided into two parts: Tier A, in which the possible fate of the pharmaceutical or its metabolites and its effects on earthworms (mortality) and plants (germination and growth) as well as the effects of the test compound on the rate of nitrate mineralization in soil are determined; and Tier B in which only effect studies are recommended for affected taxonomic levels [34-39].

The main problem associated with this approach is the fact that the no actual sales figures or measured environmental concentrations are at hand when a risk assessment is conducted. Therefore, only crude PEC calculations are performed [42]. Moreover, the (eco)toxicity tests included in Phase II focus on acute toxicity of only single compounds. Chronic and mixture toxicity is not obligatory. As the risk of an acute toxic effect from pharmaceuticals in the

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environment is unlikely and organisms in the environment are exposed to mixtures of pharmaceuticals, such limited focus results in important uncertainties. Additionally, same drugs (like sulfonamides) are used to treat both humans and animals. Although the exposures may differ, their potential effects on non-target organisms will be the same, and so the effect-testing approaches should be similar. For these reasons, many scientists have already pointed out the need for more reliable PEC and PNEC calculations for more realistic ERA of pharmaceutical [40-42].

3. Basic concepts of chemical mixture toxicology

To predict the toxicity of mixtures, ecotoxicologists use concepts originally developed by pharmacologists in the first half of the 20th century [43-48]. Since more than 20 years, they have been trying to elucidate the problem of risk assessment for complex mixtures of various substances. As a result a lot of excellent studies have been performed in this topic [49-51]. One of the main interests of scientists in the field of combination toxicology is to find out whether the toxicity of a mixture is different from the sum of the toxicities of the single compounds; in other words, will the toxic effect of a mixture be determined by additivity of dose or effect or by supra-additivity (synergism - an effect stronger than expected on the basis of additivity) or by infra-additivity (antagonism - an effect lower than the sum of the toxicities of the single compounds) The toxic effect of a mixture appears to be highly dependent on the dose (exposure level), the mechanism of action, and the target (receptor) of each of the mixture constituents. Thus, information on these aspects is a prerequisite for predicting the toxic effect of a mixture [46-47, 52].

Generally, three basic concepts for the description of the toxicological action of constituents of a mixture have been defined by Bliss and are still valid half a century later: (1) simple similar action (concentration addition, CA), (2) simple dissimilar action (independent action, IA), (3) interactions (synergism, potentiation, antagonism) [45].

Concentration addition (CA), also known as 'simple joint action', is based on the idea of a similar action of single compounds, whereas interpretations of this term can differ considerably. From mechanistic point of view, similar action means in a strict sense that single substance should show the same specific interaction with a molecular target site in the observed organisms. This is a nonintereactive process, which means that the chemicals in the mixture do not affect the toxicity of one another. Each of the chemicals in the mixture contributes to the toxicity of the mixture in proportion to its dose, expressed as the percentage of the dose of that chemical alone that would be required to obtain the given effect of the mixture. All chemicals of concern in a mixture act in the same way, by the same mechanisms, and differ only in their potencies [46-47, 52].

It has been shown that the concept of concentration addition is also applicable to nonreactive, nonionized organic chemicals, which show no specific mode of action but whose toxicity toward aquatic species is governed be hydrophobicity. The mode of action of such compounds is called narcosis or baseline toxicity [53-54]. The potency of a chemical to

induce narcosis is entirely dependent on its hydrophobicity, generally expressed by its octanol-water partition coefficient $\log K_{ow}$. As a result, in the absence of any specific mechanism of toxicity, a chemical will, within certain boundaries, always be as toxic as its $\log K_{ow}$ indicates. Mathematically, the concept of concentration addition for a mixture of n substances is described by [48]:

$$\sum_{i=1}^{n} \frac{C_i}{ECx_i} = 1 \quad (1)$$

where c_i represents the individual concentrations of the single substances present in a mixture with a total effect of x%, and ECx_i are those concentrations of the single substances that would alone cause the same effect x as observed for the mixture. According to Eq. (1), the effect of the mixture remains constant when one component is replaced by an equal fraction of an equally effective concentration of another. As an important point, concentration addition means that substances applied at less than their individual "no observable effect concentrations" (NOECs) can nevertheless contribute to the total mixture effect [46-47].

The alternative concept of independent action (IA), also known as 'independent joint action' was already formulated by Bliss [45]. IA is when toxicants act independently and have different modes of toxic action [43, 46-47]. In this case the agents of a mixture do not affect each other's toxic effect. As a result of such a dissimiliar action, the relative effect of one of the toxicants in a mixture should remain unchanged in the presence of another one. For binary mixture the combination effect can be calculated by the equitation [46]:

$$E(c_{mix}) = 1 - [(1 - E(c_1))(1 - E(c_2))] \quad (2)$$

In which $E(c_1)$, $E(c_2)$ are the effect of single substances and $E(c_{mix})$ is the total effect of the mixture. Following this equitation, a substance applied in a concentration below its individual NOEC will not contribute to the total effect of the mixture, i.e. there will be no mixture toxicity if the concentrations of all used single substances are below their NOEC [45-47, 52].

Additionally, compounds may interact with one another, modifying the magnitude and sometimes the nature of the toxic effect. This modification may make the composite effect stronger or weaker. An interaction might occur in the toxicokinetic phase (processes of uptake, distribution, metabolism, and excretion) or in the toxicodynamic phase (effects of chemicals on the receptor, cellular target, or organ).These include terms such as synergism and potentiation (i.e., resulting in a more than additive effect), or antagonism (i.e., resulting in a less than additive effect) [52]. It must be highlighted that at given concentrations of the single compounds in a mixture the combination effect will in general be higher if the substances follow the concept of concentration addition. Thus, misleadingly the different concepts were sometimes brought in correlation to the term synergism and antagonism. But synergism or antagonisms between the used substances and their effects can occur independently of a similar or dissimilar mode of action [46].

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For these reasons, prediction of the effect of a mixture based on the knowledge of each of the constituents requires detailed information on the composition of the mixture, exposure level, mechanism of action, and receptor of the individual compounds. However, often such information is not or is only partially available and additional studies are needed. In addition to considering which of these concepts should be used to evaluate combined toxic effects, the design of the study is important in quantifying the combined effects. Most of such studies are based on a comparison of observed values with those predicted by a reference mode (IA or CA). An important aspect of toxicity studies of mixtures is the impracticability of 'complete' testing. If all combinations are to be studied at different dose levels, an increasing number of chemicals in a mixture results in an exponential increase in number of test groups: to test all possible combinations (in a complete experimental design) at only one dose level of each chemical in a mixture consisting of 4 or 6 chemicals, 16 (2^4–1) or 64 (2^6–1) test groups, respectively, would be required. Such *in vivo* studies are time consuming and impossible from a practical and economical point of view. To reduce the number of test groups without losing too much information about possible interactions between chemicals, several test scenario's (statistical designs) have been proposed [52]. The study design largely depends on the number of compounds of a mixture and on the question whether it is desirable to assess possible existing interactions between chemicals in a mixture [52]:

- One approach is to test the toxicity of the mixture without assessing the type of interactions. This is the simplest way to study effects of mixtures by comparing the effect of a mixture with the effects of all its constituents at comparable concentrations and duration of exposure at one dose level without testing all possible combinations of two or more chemicals. This approach requires a minimum number of experimental groups (n + 1, the number of compounds in a mixture plus the mixture itself). If there are no dose-effect curves of each of the single compounds it is impossible to describe the effect of the mixture in terms of synergism, potentiation, antagonism, etc. This strategy would be of interest for a first screening of adverse effects of a mixture.

- The second approach is based on assessment of interactive effects between two or three compounds which can be identified by physiologically based toxicokinetic modeling, isobolographic or dose-effect surface analysis, or comparison of dose-effect curves. However, interactive effects of compounds in mixtures with more than three compounds can be best ascertained with the help of statistical designs such as (fractionated) factorial designs, ray designs or dose-effect surface analysis. Here we would like to described shortly only the isobole methods as so far they are mainly used in the studies concerning the determination of pharmaceutical mixture toxicity [25-26,46-47].

An isobole, originally developed by Loewe and Muischnek [44], is a contour line that represents equi-effective quantities of two agents or their mixtures [52]. The theoretical line of additivity is the straight line connecting the individual doses of each of the single agents that produce the fixed effect alone. The method requires a number of mixtures to be tested and is used for a graphical representation to find out if mixtures of two compounds behave in a dose-additive manner and subsequently can be regarded as chemicals with a similar

mode of action. When all equi-effect concentrations are connected by a downward concave line, the effect of the combinations is antagonistic, and a concave upward curve indicates synergism. The use of the isobole procedure to evaluate the effects of binary mixtures is widely used, but is very laborious and requires large data sets in order to produce sufficiently reliable results [52].

4. State of the knowledge concerning mixture and chronic toxicity of the residues of sulfonamides in the environment

4.1. What do we know about the long-term effects of the presence of the residues of sulfonamides in the environment?

Chronic toxicity tests are studies in which organisms are exposed to different concentrations of a chemical and observed over a long period, or a substantial part of their lifespan. In contrast to acute toxicity tests, which often use mortality as the only measured effect, chronic tests usually include additional measures of effect such as growth rates, reproduction or changes in organism behavior [55-56]. Therefore, the standard acute toxicity tests do not seem appropriate for risk assessment of pharmaceuticals, because of the nature of these compounds. The use of chronic tests over the life-cycle of organisms for different trophic levels could be more appropriate [57]. However, there is still an ongoing debate between ecotoxicologists over the determination which tests should be considered to be chronic or acute (based on their duration). This applies not only to aquatic animal testing with invertebrates and fish, but also to standard 96-h algal and 7-d higher plant test methods.

Molander et al. [19] reviewed the data published in the *Wikipharma* database – a freely available, interactive and comprehensive database on the environmental effects of pharmaceuticals that provides an overview of effects caused by these compounds on non-target organisms identified in acute, sub-chronic and chronic ecotoxicity tests. Looking at the data set as a whole, they concluded that crustaceans like *Daphnia magna* and *Ceriodaphnia dubia* were the species most commonly used (29% of all tests performed); this is hardly surprising since they are abundant and widespread, easy to keep in the laboratory, and sensitive towards a broad range of environmental contaminants. Less commonly, such tests were performed on marine bacteria *Vibrio fischeri* (12%), algae *Pseudokirchneriella subcapitata* (9.5%) and fish *Poeciliopsis lucida* (9%) and *Oncorhynchus mykiss* (8%) [19]. They have also estimated that acute tests based on microorganisms (exposure time ≤ 30 min), algae (exposure time ≤ 72 h), invertebrates (exposure time ≤ 48 h) and vertebrates (exposure time ≤ 96 h) constitute 55% of all the data compiled [19]. This information was corroborated by Santos et al. [20], who estimated that acute effects in organisms belonging to different trophic levels predominate over chronic ones in more than 60% of all the tests performed. This also concerns the available information on the ecotoxicity of sulfonamides (see Table 2).

Looking at the available acute toxicity data, it can be concluded that SAs are practically non-toxic to most microorganisms tested including selected strains of bacteria, such as *Vibrio fischeri* and *Pseudomonas aeruginosa*. However, data as are available from acute tests on the

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potential effects of SAs in the environment appear to indicate a possible negative impact on different ecosystems and imply a threat to public health. The most sensitive assays for the presence of SAs are bioindicators containing chlorophyll (algea and duckweed) [3, 22-23]. A highly toxic effect of SMX on duckweed (*Lemna gibba*) was observed. This was also supported by the results of one of our studies, where we evaluated the ecotoxicity potential of twelve sulfonamides (sulfaguanidine, sulfadiazine, sulfathiazole, sulfamerazine, sulfamethiazole, sulfachloropyridazine, sulfamethoxypyridazine, sulfamethoxazole, sulfisoxazole, sulfadimethoxine, sulfapyridine, sulfadimidine) to enzymes (acetylcholinesterase and glutathione reductase), luminescent marine bacteria (*Vibrio fischeri*), soil bacteria (*Arthrobacter globiformis)*, limnic unicellular green algae (*Scenedesmus vacuolatus*) and duckweed (*Lemna minor*). We found that SAs were not only toxic towards green algae (EC_{50} = 1.54 – 32.25 mg/L) but were even more strongly so towards duckweed (EC_{50} = 0.02 – 4.89 mg/L) than atrazine, a herbicide (EC_{50} = 2.59 mg/L) [33]. This indicates that even low concentrations of SAs may significantly affect the growth and development of plants.

However, data relating to the long-term exposure of non-target organisms, and especially how continuous exposure for several generations may affect a whole population is very limited. Most chronic toxicity data for sulfonamides, is available for invertebrates, probably because these are the briefest and therefore least expensive chronic toxicity tests to run. Available chronic toxicity data for sulfonamides is summarized in Table 3 and discussed below.

The major concern over the effects of all antimicrobials (including sulfonamides) on microbial assemblages is the development of antimicrobial resistance and the effect of this on public health. Recently, Baran et al. [3] has reviewed the papers concerning the influence of presence of SAs in the environment to antimicrobial resistance. They concluded that SAs in the environment increase the antimicrobial resistance of microorganisms and the number of bacterial strains resistant to SAs increases systematically in recent years. Resistant bacterial species commonly carried single genes, but in recent years, an increased number of pathogens that possess three SAs-resistant genes have been observed. Moreover, they have also highlighted that these drugs have shown the highest drug resistance, almost twice as high as tetracyclines and many times higher than other antibiotics. Most often, bacterial resistance to SAs has been described in *Escherichia coli*, *Salmonella enterica* and *Shigella spp*. from the manure of farm animals, from meat and from wastewater [3]. The implications of antimicrobial resistance for aquatic ecosystem structure and function remain unknown, but the human health implications of widespread resistance are of clear concern [55].

Additionaly, Heuer and Smalla [58] investigated the effects of pig manure and sulfadiazine on bacterial communities in soil microcosms using two soil types. In both soils, manure and sulfadiazine positively affected the quotients of total and sulfadiazine-resistant culturable bacteria after two months. The results suggest that manure from treated pigs enhances spread of antibiotic resistances in soil bacterial communities. Monteiro and Boxall [59] have recently examined the indirect effects of sulfamethoxazole on the degradation of a range of human medicines in soils. It was observed that the addition of SMX significantly reduce the rate of degradation of human non-steroidal anti-inflammatory drugs, naproxen. This observation

may have serious implications for the risks of other compounds that are applied to the soil environment such as pesticides.

Substance name	Type of organism	Acute toxicity	Chronic toxicity	Ref.
SDZ	*Daphnia magna*	$EC_{50, 48h}$ = 221 mg L^{-1} (166 – 568 mg L^{-1})	$EC_{50, 21d}$ = 13.7 mg L^{-1} (12.2 – 15.3 mg L^{-1})	[60]
SMX	*Brachionus calyciflorus*	$EC_{50, 24h}$ = 26.27 mg L^{-1} (16.32 – 42.28 mg L^{-1})	$EC_{50, 48h}$ = 9.63 mg L^{-1} (7.00 – 13.25 mg L^{-1})	[29]
	Clathrina dubia	$EC_{50, 48h}$ = 15.51 mg L^{-1} (12.97 – 18.55 mg L^{-1}	$EC_{50, 7d}$ = 0.21 mg L^{-1} (0.14 – 0.39 mg L^{-1})	
		-log$EC_{50, 15 min}$	-log$EC_{50, 24h}$	
SDMD		3.12 (± 0.04) M	4.08 (± 0.06) M	
SPY		2.92 (± 0.05) M	3.84 (± 0.04) M	
SMX		3.32 (± 0.04) M	4.45 (± 0.05) M	
SDZ	*Photobacterium phosphoreum*	3.32 (± 0.02) M	4.50 (± 0.06) M	[61]
SSX		3.81 (± 0.02) M	4.43 (± 0.03) M	
SMM[a]		3.67 (± 0.03) M	5.05 (± 0.05) M	
SCP		4.30 (± 0.04) M	4.78 (± 0.04) M	
SQO	*Daphnia magna*	$EC_{50, 48h}$ = 131 mg L^{-1} (119 – 143 mg L^{-1})	$EC_{50, 21d}$ = 3.466 mg L^{-1} (2.642 – 4.469 mg L^{-1})	[25-26]
SGD		$EC_{50, 48h}$ = 3.86 mg L^{-1} (3.19 – 5.08 mg L^{-1})	$EC_{50, 21d}$ = 0.869 mg L^{-1} (0.630 – 1.097 mg L^{-1})	
SDMD		$EC_{50, 48h}$ = 202 mg L^{-1} (179 – 223 mg L^{-1})	$EC_{50, 21d}$ = 4.25 mg L^{-1} (3.84 – 4.62 mg L^{-1})	
SDMD	*Daphnia magna*	$EC_{50, 48h}$ = 215.9 mg L^{-1} (169.6 – 274.9 mg L^{-1})	$EC_{50, 21d}$ no effect up to 30 mg L^{-1}	[16]
STZ		$EC_{50, 48h}$ = 616.9 mg L^{-1} (291.7 – 1303.6 mg L^{-1})	LOEC = 35 mg L^{-1}	
SDMD	*Moina macrocopa*	$EC_{50, 48h}$ = 110.7 mg L^{-1} (89.5 – 136.9 mg L^{-1})	$EC_{50, 8d}$ no effect up to 35 mg L^{-1}	
STZ		$EC_{50, 48h}$ = 391.1 mg L^{-1} (341.9 – 440.3 mg L^{-1})	no effect up to 30 mg L^{-1}	

[a]Sulfamonometoxine

Table 3. An overview of the available information on the chronic toxicity of sulfonamides to different organisms

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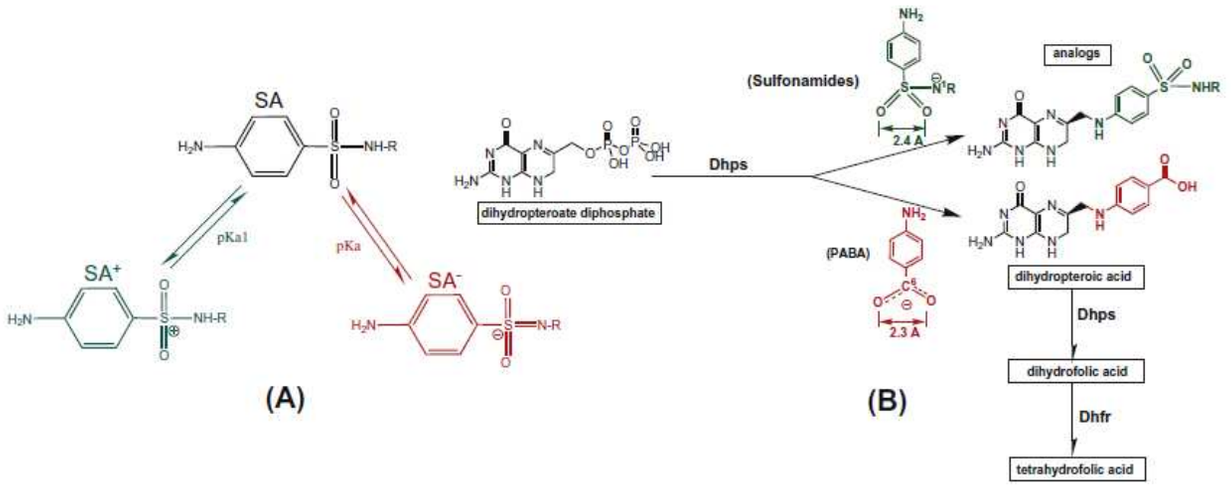

Figure 1. (A) Scheme of SA ionization in equilibrium, (B) Mechanism for synergistic effect between SA and TMP in bacterium (adopted from Reference [61])

Only few studies have also explored effects of SAs on aquatic microbes. It must be highlighted that it was already proved that the effects of antibiotics like SAs on bacteria should not be determined using acute tests. These compounds possess specific mode of action and impacts frequently became evident upon extending the incubation period. Most of the toxicity data available for *Vibrio fischeri* using short exposure times (between 5 and 30 min) rather than a 24 h exposure show that SAs have a low toxic potential in this respect, because these compounds interfere only slightly with biosynthetic pathways. Toxicity tests with bacteria have shown that chronic exposure to antibiotics is crucial rather than acute [14,50, 62-63]. This is also supported by the results of [30,61,64]. The toxicity of sulfadimidine (sulfamethazine) in standard 15 min acute test with this luminescent bacteria obtained EC_{50} was 344.7 mg L^{-1} [30] but in 18 h test its toxicity was in the range of 3.68 – 4.57 mg L^{-1} depending on the type of strain of these marine bacteria [64]. Also Zou et al. [61] determine the chronic (24 h exposure) and acute (15 min exposure) toxicity to *Photobacterium phosphoreum* for seven SAs. These experiments revealed that sulfachloropyridazine (SCP) was more toxic than other SAs, whereas sulfapyridine (SPY) was relatively less toxic than other SAs (see Table 3). The order of acute toxicity was as follows: SCP > SSX > SMX > SMM > SDZ > SDMD > SPY. However, the order of chronic toxicity was different: SMM > SCP > SDZ > SMX > SSX > SDMD > SPY. Clearly, different order of toxicity between the acute and chronic exposure indicated a different toxicity mechanism (see Fig. 1). It has been reported that the acute toxic effects of pollutants to *P. phosphoreum* are caused by interfering LUC-catalyzed bioluminescent reaction and therefore LUC was found to be the receptor protein for the antibiotics. In contrast, the receptor for the antibiotics in the chronic toxicity test was dihydropterinic acid synthetase (DHPS).

Studies conducted on the toxicity mechanism of single SAa indicated that the pKa played a vital role in the toxic effect of SAs or their antibacterial activity [32]. Because LUC (Lucyferase) is an endoenzyme, and SAs have to be transported into the cell before bind with LUC, it was clear that the antibiotic toxicity included both LUC-binding and a toxic transportation effect

(which can be described using pKa). pKa is a decisive factor in transporting SAs into the cell. Three species (neutral, cationic and anionic) of SAs depend on the pKa and surrounding pH values. The neutral species have higher cell membrane permeability than anionic species. Therefore, pKa was the key parameter of sulfonamides toxic effects. Some similarity in acute and chronic toxicity mechanisms was observed. However, in conclusion the distinct receptor proteins of SAs in acute toxicity and chronic toxicity led to the different toxicity mechanisms of single antibiotics [61]. A comparison of the results of short and long term bioassays with *Vibrio fischeri* demonstrates the risk of underestimating the severe effects of substances with delayed toxicity in acute tests.

Similar conclusions can be obtained if only acute toxicity of SAs to invertebrates is taken into consideration. Detailed information is presented in Table 3. No acute effects on *D. magna* were observed in the investigation of Wollenberger et al. [60]. However, reproductive effects (EC_{50}) were observed for sulfadiazine in the range of 5 to 50 mg L^{-1}. This drug caused mortality in the parent generation during the long-term (3 weeks) exposure. Such results suggest that crustacean reproduction test should be included in the test strategy [60]. Similar correlation was also found by Isidori et al. [29] who investigated the acute and chronic toxicity of SMX to *B. calciflorus* (24 h and 48 h exposure) and *C. dubia* (48 h and 7 d exposure time). As expected chronic tests showed higher toxicity that acute tests. Also Park and Choi [16] evaluated the acute and chronic aquatic toxicities of four SAa using standard tests with *D. magna* and *M. macrocopa*. The results from the chronic toxicity tests in this study showed that sensitivity of *M. macrocopa* was similar to that of *D. magna.* However, the exposure duration for *M. macrocopa* was only 8 days whereas for D. magna was 21 days. *Moina* shares many characterisitcs with *D. magna* (e.g. large population densities, high population growth rates, short generation time, and easiness of culture) and is often preferred for hazard evaluation because of its relatively short life span and wide geographical distribution. However, Park and Choi found no significant effects on reproduction of *D. magna* at concentrations of SMZ up to 30 mg L-1. In contrast De Liguoro et al. [25-26] observed strong inhibitory effect of SMZ on reproduction of *D. magna* (nearly 100% inhibition with SMZ at a concentration of 12.5 mg L-1). This could be explained by that fact that in the Park and Choi study, daphnids were fed daily not only with algae, but also with the EPA recommended YCT that contains yeast, a known good natural source of folate [16]. Eguchi et al. [28] have shown that SAs interfere with folate synthesis in green algae. Therefore, this supplement of folic acid may well have protected the reproduction of the test organisms by compensating for the deficiencies caused by SMZ. Generally, when testing antibacterials on *D. magna*, effects on the reproductive output occur at concentrations which are at least one order of magnitude below the acute toxic levels [60].

Unfortunately, there is no information about long-term effects of the residues of these compounds to higher plants and other aquatic as well as terrestrial organisms. Therefore, it seems to be necessary for researchers to study the chronic toxicity of antibiotic [46-47, 55] because of their widespread use and continuous emissions into the environment [14].

4.2. What do we know about mixture toxicity of the residues of sulfonamides in the environment?

As SAs occur in natural environment not as a single, isolated drug but usually together with other compounds of the same family or the same type, accumulated concentrations or synergistic-antagonistic effects need to be considered. Sulfonamides are widely used in combination therapy together with their potentiator (mostly trimethoprim, TMP) in human and veterinary medicine [1-3]; thus, the occurrence of TMP together with other antibiotics has been commonly detected [3].

Santos et al. [20] pointed out that, ecotoxicological data show that the effects of mixtures may differ from those of single compounds. For example, Cleuvers [46] showed that a mixture of diclofenac and ibuprofen exhibited a greater than predicted toxicity to *D. magna*, and that the addition of two more drugs increased the toxicity towards the test species even further. Available mixture toxicity data for sulfonamides is summarized in Table 4 and discussed below.

Substance name/ Mixture composition	Test scenario (statistical design)	Test description (organism, test duration)	Toxicity of single compounds ($EC_{50single}$)	Mixture toxicity ($EC_{50mixture}$)	Conclusions	Ref.
SDM	Evaluation of the toxicity of the mixture of selected compounds based on concentration addition concept.	*Selenastrum capricornutum*, according to OECD 201	2.30 mg L^{-1}		Synergistic growth inhibition between SAs and TMP or PMT and for SMX:AcSMX:TMP mixture.	[28]
SMX			1.50 mg L^{-1}			
SDZ			2.19 mg L^{-1}			
Trimethoprim (TMP)			80.8 mg L^{-1}			
Pyrimethanine (PMT)			5.06 mg L^{-1}			
AcSMX			>100mg L^{-1}			
AcSDM			>100mg L^{-1}			
AcSDZ			>100mg L^{-1}			
SMZ + TMP				0.275 mg L^{-1}		
SDA + TMP				0.465 mg L^{-1}		
SDM + PMT				2.36 mg L^{-1}		
SMX:AcSMX:TMP (20:105:3)				0.784 mg L^{-1}		
SDM:AcSDM:TMP (176:8:1)				2.17 mg L^{-1}		
SDZ:AcSDZ:TMP (42:24:1)				2.08 mg L^{-1}		
SDZ	Assessment of interactive effects between two compounds identified by	*Daphnia magna*, 48 h	212 mg L^{-1}		Antagonistic interaction for mixtures: SMZ + SDZ SMZ + SGD SMZ + SMR	[26]
SGD			3.86 mg L^{-1}			
SMR			277 mg L^{-1}			
SDM			202 mg L^{-1}			
SDMD (SMZ)			270 mg L^{-1}			

SQO TMP			131 mg L^{-1} 149 mg L^{-1}	SMT + SDM Complex interaction (synergism additivity and antagonism) for mixture of SMT + SQO Simple additivity for SMZ + TMP	
binary mixtures of SMZ +6 compounds	isobologram method.				
SGD SQO SGD SQO	Assessment of interactive effects between two compounds identified by isobologram method.	*Daphnia magna*, 21 d *Pseudokirchneriella subcapitata*, 96 h	0.896 mg L^{-1} 3.466 mg L^{-1} 43.559 mg L^{-1} 0.246 mg L^{-1}	Additive (antagonistic) interaction between SQO and SGD.	[25]
			Toxicity of single compound (-log$EC_{50,\ 15\ min}$)	Toxicity of binary mixture SAs and TMP(-log$EC_{50,\ mixture}$)	
SDMD	Evaluation of the toxicity of the mixture of selected compounds based on concentration addition concept	*Photobacterium phosphoreum*, 15 min and 24 h	3.12 (± 0.04) M	2.78 (± 0.02) M	Antagonistic interaction between SAs and TMP in acute toxicity test.	[61]
SPY			2.92 (± 0.05) M	2.89 (± 0.02) M		
SMX			3.32 (± 0.04) M	2.79 (± 0.01) M		
SDZ			3.32 (± 0.02) M	2.76 (± 0.03) M		
SSX			3.81 (± 0.02) M	2.79 (± 0.07) M		
SMM			3.67 (± 0.03) M	2.73 (± 0.02) M		
SCP			4.30 (± 0.04) M	3.00 (± 0.03) M		
TMP			3.22 (± 0.07) M			
			Toxicity of single compound (-log$EC_{50,\ 24h}$)	Toxicity of binary mixture SAs and TMP (-log$EC_{50,\ mixture}$)		
SDMD			4.08 (± 0.06) M	5.08 (± 0.05) M		
SPY			3.84 (± 0.04) M	4.85 (± 0.07) M		
SMX			4.45 (± 0.05) M	5.50 (± 0.07) M	Synergistic interaction between SAs and TMP in chronic toxicity test.	
SDZ			4.50 (± 0.06) M	5.42 (± 0.03) M		
SSX			4.43 (± 0.03) M	5.45 (± 0.03) M		
SMM			5.05 (± 0.05) M	6.01 (± 0.05) M		
SCP			4.78 (± 0.04) M	5.73 (± 0.05) M		
TMP			5.37 (± 0.02) M			

Table 4. An overview of the available information on the mixture toxicity of sulfonamides to different organisms

The toxicity of mixture of sulfonamides to non-target organisms was firstly reported by Brain et al. [22] and Eguchi et al. [28]. Brain et al. investigated the toxicity of the mixture of eight

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most commonly used pharmaceuticals belonging to different groups (atorvastatin, acetaminophen, caffeine, sulfamethoxazole, carbamazepine, levofloxacin, sertraline and trimethoprim) to the aquatic macrophytes *Lemna gibba* and *Myriophyllum sibircu*m. Given the diversity in mode of action of these compounds, the toxicity of the mixture in the microcosms was likely via response addition. Generally, both species displayed similar sensitivity to the pharmaceutical mixture [22].

On the other hand, Eguchi et al. [28] found that a mixture of trimethoprim or pyrimethamine (pyrimethamine is often used as a substitute for trimethoprim), sulfamethoxazole and sulfadiazine significantly increased growth inhibition (synergistic effect of the mixture was observed) in the algae *S. capricornutum*. To investigate the synergistic influence of combined drugs on the growth of green algae, SAs and TMP or PMT (TMPs) were simultaneously added to *S. capricornutum* culture. In this experiment, the concentration of TMPs was fixed at the no observed effect concentration (NOEC) and the concentration of the SAs were altered. These combined drugs are frequently used in the veterinary field in many countries. Combination of SMZ and SDA with TMP rendered the growth inhibitory activity significantly increased in comparison with their individual activities (see Table 4 and Fig. 2(A)). On the contrary, combination of SDM with PYR did not show such an effect. Moreover, as SAs are thought to be partly metabolized to AcSAs in the bodies of animals, Eguchi et al. [28] have also tested the toxicity of the mixture of SAs their metabolites and TMP. Therefore the test of combined drugs was done by using the combinations corresponding AcSAs at a ratio according to the concentrations detected in the urine of pigs fed with SAs. The ratio was SMZ:AcSMZ:TMP = 20:105:3, SDM:AcSDM:PMT = 167:8:1 and SDA:AcSDA:TMP = 42:24:1. A similar synergistic effect to that described above was observed with combinations of SMZ, TMP, and AcSMZ (see Table 4). However, combination of SDM or SDA with their acetylate and PMT or TMP did not show a synergistic effect on growth in excretion ratio. A reason must be that the concentration of TMP used was not enough to express synergistic influence in combination with SDM or SDA. These results indicate that several combined drugs that show a synergistic effect *in vitro* may have an actual synergistic effect on algae in ecosystem although excretion ratio can vary in animal condition or other factors. The synergistic effect observed by the combination of SAs and TMPs in this study indicates that the simultaneous release of several antimicrobial agents may result in greater toxicity to microorganisms in the environment than the release of the same agents individually. Furthermore, the rate of growth inhibition by SAs by addition of folic acid was investigated in this study. It was observed that the growth inhibitory activity of the combination of SDA and TMP was significantly reduced by the addition of 20 ng/l of folic acid to the medium. Significantly, folic acid exhibited a similar effect when SDA was tested alone, but not when TMP was tested alone (see Fig. 2(B)) [28].

Both SAs and TMPs inhibit the folate synthesis pathway in bacteria, but their inhibition sites are different. SAs inhibit dihydropterinic acid synthetase (DHPS), thereby inhibiting the synthesis of folic acid. On the other hand, TMPs inhibits dihydrofolic acid reductase (DHFR), which converts folic acid to 7,8-dihydrofolic acid (7,8- DHF) and 5,6,7,8-tetrahydrofolic acid (5,6,7,8-THF), both active forms of folic acid suitable for utilization. Therefore, the synergistic effect of the combination of SAs and TMPs is likely to be due to the cumulative effect of their

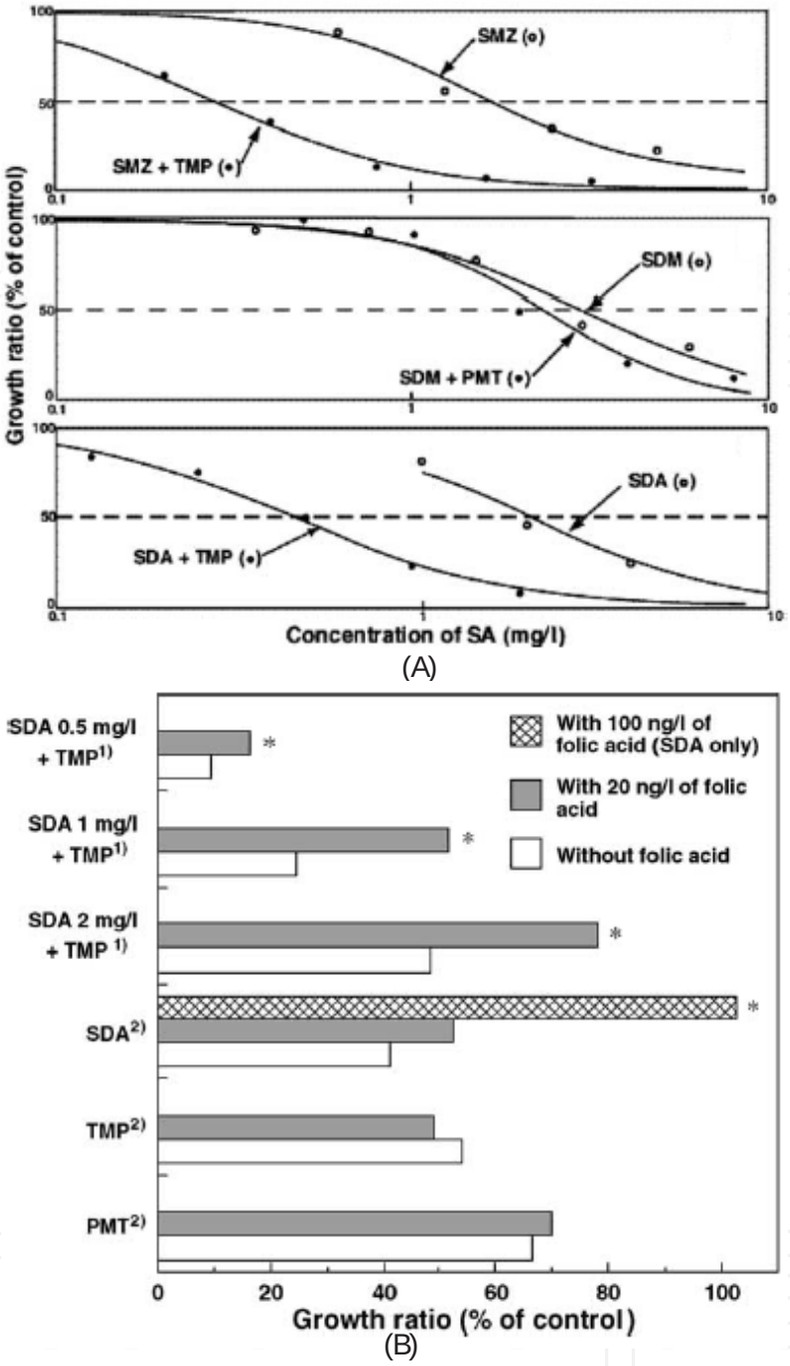

Figure 2. (A) The dose-response curve of SAs (SMT – sufamethzine, SDA – sulfadiazine, SDM – sulfadimethoxine) combined with TMP (trimethoprim) or PMT (pyrimethamine); **(B)** Recovery of growth inhibition be addition of folic acid (*observed siginifcant difference to negative control – without folic acid, [1)] concentration of SDA (sulfadiazine) in combination, TMP was used at the NOEC, [2)] used at the EC_{50} concentration) (adopted from References [28])

actions on two different sites in the folate biosynthesis pathway. Since SAs block the synthesis of folate, the growth inhibitory effect of this compound can be reversed by the addition of folate. In contrast, TMP blocks enzymes downstream of folate in the synthesis pathway, thus addition of folate will not reverse the growth-inhibiting effect of this compound. Since algea

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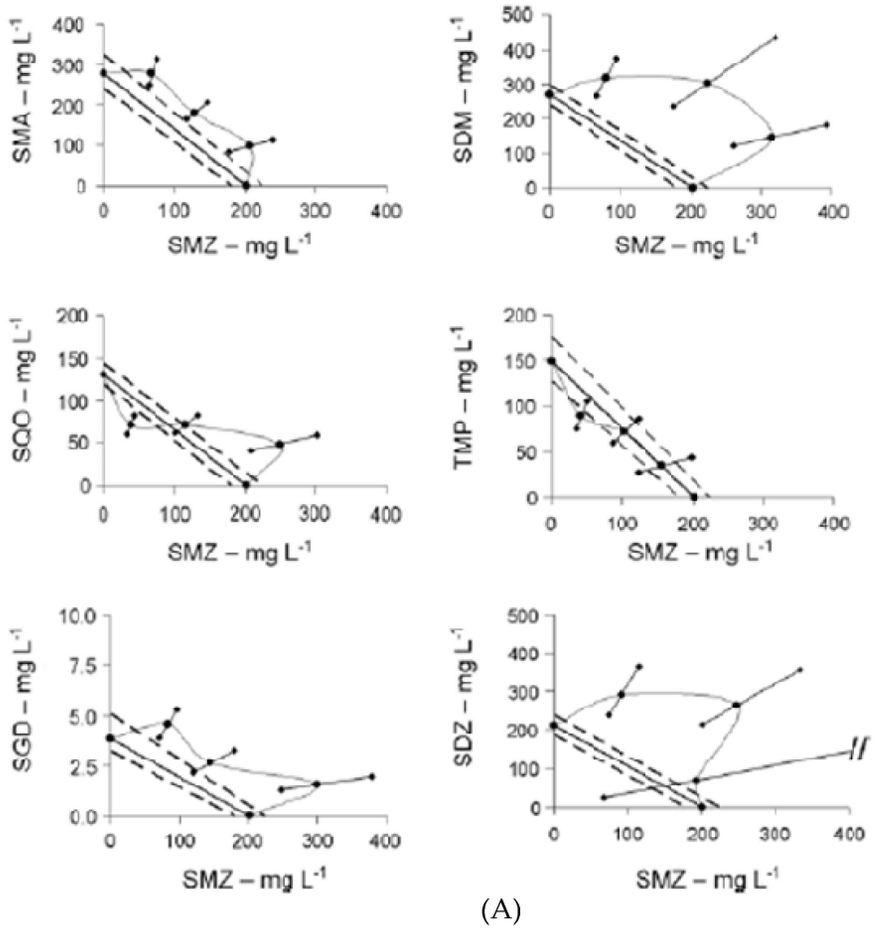
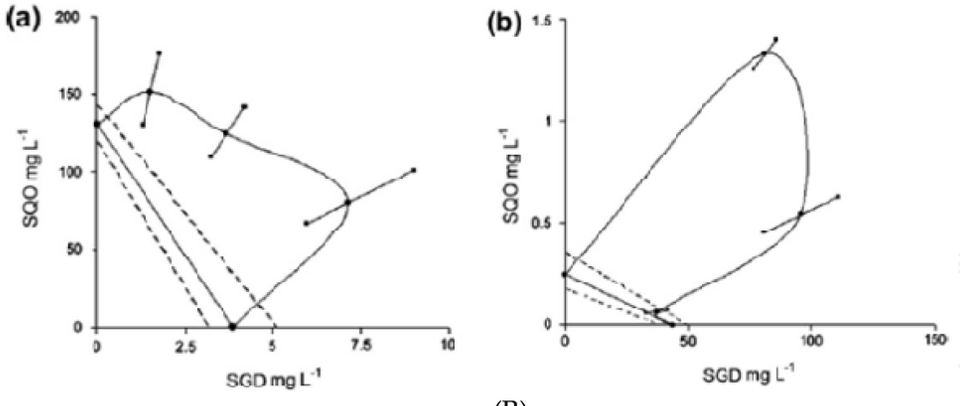
also have a similar folate synthesis pathway, the growth inhibitory effect of SAs on these organisms is likely to be the result of the same inhibitory mechanism. Therefore, algal cells could survive in the presence of SAs, but not TMP, when folic acid was added to the medium.

De Liguoro et al. [25-26] evaluated the acute mixture toxicity of combining sulfamethazine with TMP towards *D. magna* and effects of different mixtures of sulfaquinoloxine (SQO) and sulfaguanidine (SGD) on *D. magna* and *P. subcapitata* (see Table 4). The additive toxicity of these compounds was evaluated using the isobologram method. In Fig. 3A, the isoboles showing the different type of combination effects are presented. Taking into account confidence intervals SMZ showed infra-additivity when paired with SDZ, SGD, SMA or SDM. When SMZ was paired with SQO the interaction was more complex, as each type of combination effects (supra-additivity, additivity and infra-additivity) was observed at the three different combination ratios. Simple additivity was recorded when SMZ was combined with the sulfonamide-potentiator TMP (Fig. 3A). Tests with paired SQO and SGD were based on the individual EC_{50} (for *D. magna* see [25]). In each paired test, the concentration–response relationship was analyzed for three selected combination ratios equidistantly distributed on the additivity line. In Fig. 3B, the isoboles based on the effects of different mixtures of SQO and SGD on *D. magna* and *P. subcapitata* are depicted. Only in one test, where relatively low concentrations of SQO were combined with relatively high concentrations of SGD on *P. subcapitata*, the two paired compounds showed simple additivity. In all the other tests a less than additive (antagonistic) interaction was detected. In this study, binary tests confirmed the tendency of SAs mixtures to act less than additively. So, in general terms, it seems sufficiently precautionary to consider their environmental toxicity as additive. However, when combining SQO and SGD on P. *subcapitata*, the obtained asymmetric isobologram shows that the interaction is mixture-ratio dependent, a phenomenon already observed when mixtures of SQO and sulfamethazine were tested on *D. magna* [26].

Zou et al. [61] have recently highlighted that these results cannot represent the mixture toxicity between the SAs and TMP in an actual environment because non-target organisms (microlage and *D. magna*) were used in these studies. Bacterium is typically the target-organism of an antibiotic, and thus in their opinion, a bioassay with *Photobacterium phosphoreum* is a more reliable tool to determine the toxicity of various antibiotics. Moreover, most studies focus on the acute mixture toxicity. Therefore, in their study they have: determined not only the acute (15 min exposure) but also chronic (24 h exposure) toxicity *to P. phosphoreum* for single SA and their potentiator, and for their mixtures (SA with TMP); evaluated the differences between chronic and acute mixture toxicity; and revealed the difference between their toxicity mechanisms by using QSAR models. A comparison of chronic vs. acute mixture toxicity revealed the presence of an interesting phenomenon, that is, that the joint effects vary with the duration of exposure; the acute mixture toxicity was antagonistic, whereas the chronic mixture toxicity was synergistic. Based on the approach of QSARs and molecular docking, this phenomenon was proved to be caused by the presence of two points of dissimilarity between the acute and chronic mixture toxicity mechanism: (1) the receptor protein of SAs in acute toxicity was LUC, while in chronic toxicity it was DHPS, and (2) there is a difference between actual concentration of binding-LUC in acute toxicity and individual binding-DHPS in chronic toxicity (see Fig. 1). The existence of these differences poses a challenge for the assessment of routine combinations in medicine, risk assessment, and mixture pollutant control, in which, previously, only



(A)



(B)

Figure 3. (A)*D. magna* immobilization test: 48 h EC_{50} isobolograms of SMZ paired with other SAs and TMP at three selected combination ratios; **(B)** isobolograms of paired SQO and SGD *in D. magna* immobilization test (adopted from References [25-26])

a synergistic effect has been observed between SA and their potentiator. The toxicity effect of mixtures is associated with the transportation of toxic effects of individual chemicals into cells, the interaction toxic effects of individual chemical-binding-receptor proteins. According to

acute toxicity mechanism of single antibiotics it can be concluded that the transportation toxic effect is highly related to pKa values and the interaction toxic effect can be described by LUC-SAs binding or LUC-TMP binding. The synergistic effect between SAs with TMP has also been observed in the field of medicine and proved to be caused by the blocking of synthesis of folic acid. First SAs inhibit DHPS, which catalyzes the formation of dihydropteroic acid. Then TMP inhibits DHFR, which catalyzes the formation of tetrahydrofolic acid from dihydrofolic acid. In an acute mixture toxicity there were more SAs-binding-LUC and TMP-binding-LUC. However, in chronic mixture toxicity, the concentration of SAs-binding-DHPS was less compere to TMP-binding-DHFR. It can therefore be concluded that the dissimilarities in the concentrations of individual chemical-binding receptor proteins also lead to the different joint effect (SA with TMP) in acute and chronic mixture toxicity [61].

These examples highlight the fact that the simultaneous presence of several pharmaceuticals in the environment may result in a higher level of toxicity towards non-target organisms than that predicted for individual active substances. More ecotoxicological studies should therefore be done to evaluate the impact of different mixtures of pharmaceuticals in non-target organisms.

5. Conclusions

The reason for concern regarding risks of mixtures is obvious. Man is always exposed to more than one chemical at a time. This dictates the necessity of exposure assessment, hazard identification, and risk assessment of chemical mixtures. However, for most chemical mixtures data on exposure and toxicity are fragmentary, and roughly over 95% of the resources in toxicology is still devoted to studies of single chemicals. Moreover, organisms are typically exposed to mixtures of chemicals over long periods of time; thus, chronic mixture toxicity analysis is the best way to perform risk assessment in regards to organisms.

However, testing of all kinds of (complex) mixtures of chemicals existing in the real world or of all possible combinations of chemicals of a simple (defined) mixture at different dose levels is virtually impossible. Moreover, even if toxicity data on individual compounds are available, we are still facing the immense problem of extrapolation of findings obtained at relatively high exposure concentration in laboratory animals to man being exposed to (much) lower concentrations.

As stated by several authors, it is essential to investigate if mixtures of pharmaceuticals interact, leading to a larger effect in the environment than would be predicted when each compound is considered individually. Mixtures with antibiotics in the environment may be very complex (e.g. wastewater effluent) but they also may be simple. Although the latter may be more easily studied experimentally, in both cases the identification and quantitative description of synergism caused by specific substances is crucial.

Over past 10 years there has been increasing interest in the impacts of SAs and other veterinary medicines in the environment and there is now a much better understanding about their environmental fate and their impacts on aquatic and terrestrial organisms. However, there are still a number of uncertainties that require addressing before there can be a full understanding of the environmental risks of these compounds. Areas requiring further research are presented below.

- The assessment of the potential impacts of those SAs for which ecotoxicity data is lacking but are seen to regularly occur in the environment.
- More information about the ecotoxicity of these compounds to soil organisms should be provided. This regards to acute, chronic and single/mixture toxicity of most of the veterinary pharmaceuticals.
- Information on the potential environmental effects of parent compounds (drugs) as well as metabolites and transformation products. This includes the single and joint effects evaluation.
- Further research is required on the mixture toxicity of SAs in combination with other medicines and non-medicinal substances.
- The possible indirect effects of SAs should be identified.
- Data from acute and chronic ecotoxicity tests on species belonging to different trophic levels such as bacteria, algea, crustaceans and fish among others, is relevant to illustrate the several adverse effects that environmental exposure to measured concentrations of these contaminants can have. The principal toxicological endpoints/studies that are described are growth, survival, reproduction and immobilization of species, comparatively to trangenerational and population level studies that are still sparse. In the near future, the evaluation of chronic toxicity effects should be set out as a priority for the scientific community since simultaneous exposure to pharmaceuticals, metabolites and transformation products of several therapeutic classes are unknown and whose probable effects on subsequent generations should be assumed.

Abbreviations

Abbreviation	Full name
CA	Concentration Addition
DHFR	Dihydrofolic Acid Reductase
DHPS	Dihydropterinic Acid Synthetase
EPA	Environmental Protection Agency
ERA	Environmental Risk Assessment
IA	Independent Action
LUC	Lucyferase
MEC	Measured Environmental Concentration
NOEC	No Observable Effect Concentrations
PABA	*p*-aminobenzoic acid

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Abbreviation	Full name
PEC	Predicted Environmental Concentration
PMT	Pyrimethamine
PNEC	Predicted Non-Effective Concentrations
RQ	Risk Quotient
SAs	Sulfonamides
SCP	Sulfachloropyridazine
SDM	Sulfadimethoxine
SDMD (SMZ)	Sulfadimidine (Sulfamethazine)
SDZ	Sulfadiazine
SGD	Sulfaguanidine
SMP	Sulfamethoxypyridazine
SMR	Sulfamerazine
SMTZ	Sulfamethiazole
SMX	Sulfamethoxazole
SPY	Sulfapyridine
SQO	Sulfaquinoxaline
SSX	Sulfisoxazole
STZ	Sulfathiazole
TMP	Trimethoprim

Table 5. List of abbreviations used in the text

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