Antibacterial activity of sulfamethoxazole transformation products (TPs): General relevance for sulfonamide TPs modified at the *para*-position

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Abstract

Sulfonamide antibiotics undergo transformation in the aquatic environment through biodegradation, photolysis or hydrolysis. In this study, the residual antibacterial activity of 11 transformation products (TPs) of sulfamethoxazole (SMX) were investigated with regard to their *in vitro* growth and luminescence inhibition on *vibrio fischeri* (30 min and 24 h exposure). The two transformation products 4-hydroxy-SMX and N^4-hydroxy-acetyl-SMX were synthesized in-house and confirmed by NMR and HRMS. Results of individual compound experiments showed that TPs modified at the *para*-position still exhibit clear antibacterial effects, while TPs resulting from breakdown of the SMX structure lost this mechanism of action. 4-NO_2- and 4-OH-SMX were found to inhibit growth at a clearly higher level than the parent compound SMX. In contrast, the N^4-acetyl- and N^4-hydroxy-acetyl-derivatives retain less than 10% and 5% of the effect of SMX on growth and luminescence inhibition, respectively. The effect of a mixture of *para*-modified TPs was observed to be additive. Considering the homologous series of sulfa drugs widely prescribed and their common mechanism of action, the potential environmental impact must consider for the total amount of sulfonamide antibiotics and their derivative TPs, which might end up in a water bodies. Extrapolating the results obtained here for the *para*-TPs of SMX to other sulfa drugs and determining the persistence and occurrence of these compounds in the aquatic environment is required for improved risk assessment.

Keywords: growth inhibition, intermediates, metabolites, sulfa drugs, ecotoxicity

Introduction

Residues of pharmaceuticals and personal care products (PCPPs) are ubiquitously detected in urban wastewater and surface water alike (Herberer, 2002). It recently has been demonstrated that PCPPs can adversely act upon freshwater biofilms by respiration suppression, growth inhibition and community alteration with momentarily unknown consequences for higher trophic levels and stream ecosystem functioning (Rosi-Marshall et al., 2013). In the context of the ongoing discussion about the risk assessment of the manifold transformation products (TPs) being formed, the question was raised whether TPs still exhibit the targeted mechanisms of action of the parent compound (De Bel et al., 2009; Escher and Fenner, 2011; Wammer et al., 2011). In view of the frequent occurrence of sulfonamide antibiotics and due to their antimicrobial nature, the present study aims to address the (residual) ecotoxicological effects of sulfamethoxazole TPs. Sulfamethoxazole (SMX) is the most prominent short-acting representative of sulfonamide antibiotics used in high amounts in human and veterinary applications to treat and prevent bacterial infections of both Gram positive and Gram negative species. It can be regarded as ubiquitous in urban wastewaters and is frequently detected in surface water (Tamtam et al., 2008; Watkinson et al., 2009).

The bacteriostatic effect of sulfonamides on cell reproduction originates from the sulfanilamide toxicophore (sulfonamide group bound to aniline in the *para*-postion, Figure 1) and is based on competitive enzyme inhibition and metabolic interference due to its similarity in molecular structure to *p*-aminobenzoic acid (*p*ABA) – an essential carboxylic acid involved in the natural intracellular folic acid synthesis of bacteria.

A number of recent studies reported different chemical or biological SMX TPs formed in aquatic systems at lab-scale such as biodegradation, photolysis or hydrolysis (Bonvin et al., 2012; García-Galan et al., 2008; Larcher and Yargeau 2011, 2012; Nödler et al., 2012; Müller et al., 2013). However, while the ecotoxicity and identification of SMX TPs formed during wastewater ozonation receives increased attention (Dodd et al. 2009; Larcher and Yargeau 2013), only limited information is available regarding the risk assessment of TPs from conventional natural transformation reactions. Additionally, sulfonamide TPs are rarely included in routine monitoring campaigns, which is why only scarce data is available about their occurrence.

Some of the TPs reported for SMX still contain a toxicophore-like moiety and some derivatives incorporate almost the complete SMX parent compound structure. As a consequence, it is relevant and essential to determine the residual antibacterial activity of the manifold SMX TPs.

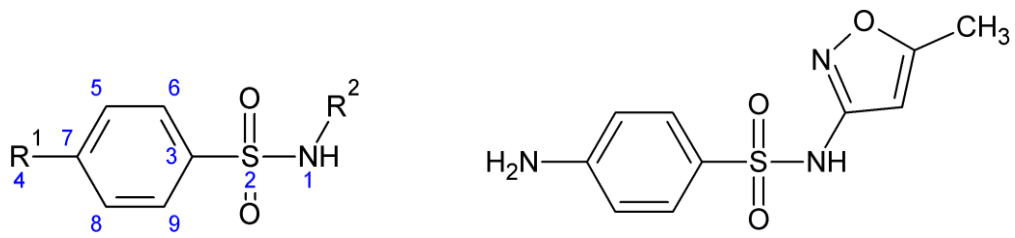


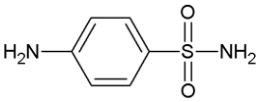
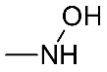
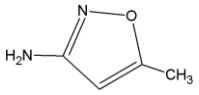
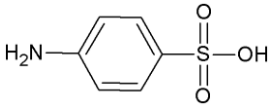
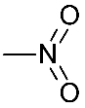
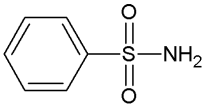
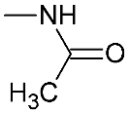
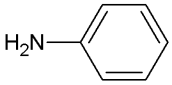
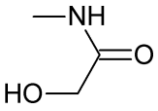
Figure 1. Generic structure, toxicophore (with R_1 = NH_2) and atom numbering of sulfonamide antibiotics (left) and structure of sulfamethoxazole (right).

Previous studies on micropollutants revealed that in most cases the TPs are less toxic than the parent compound (Boxall et al., 2004). However, some TPs exhibited similar or higher ecotoxicological effects than the parent compound and hence, experimental testing remains indispensable. Moreover, with regard to the current concerns about antibiotic resistance in microbial biofilms (Schwartz et al., 2003), the need to investigate the possible residual antibacterial potential of sulfonamide antibiotic TPs is apparent.

Addressing this need of better assessment of the potential for sulfa drugs TPs, the present study screened 11 TPs of SMX (Table 1) formed during human metabolism, microbial biodegradation, photolysis and hydrolysis for their toxicity. TPs formed by the decomposition of SMX are referred to here as breakdown products, while TPs formed by transformations limited to the *para*-position are denoted as derivatives. Formation processes reported in literature and physico-chemical properties for the selected SMX TPs are given in Table S1 (Supporting Information). The two metabolites N^4-hydroxy-acetyl-SMX and 4-hydroxy-SMX were synthesized in-house as they were not commercially available. These were included in the study since they have been suggested to occur from biotransformation with activated sludge bacteria (Larcher and Yargeau, 2011; Gauthier et al., 2010). As bioassays to investigate the effect of the TPs, luminescence inhibition (LI) experiments were selected with LI as a very sensitive and rather non-specific endpoint. Tests were carried out providing growth medium, which allows in parallel to evaluate the bacteriostatic mechanism of action of sulfonamides by growth inhibition (GI) determination as the second endpoint.

The three research hypotheses considered in this study were: i) SMX TPs retaining a sulfonamide toxicophore-like moiety exhibit residual antibacterial properties and these can be related to their physico-chemical properties, ii) TPs not exhibiting the toxicophore are ecotoxicologically relevant via another mechanism of action, iii) these trends can be generalized to other homologous sulfonamide antibiotics to anticipate ecotoxicologically relevant TPs.

Table 1. Structures and abbreviations of sulfamethoxazole transformation products; [a] synthesized in this study

Derivatives (R^1 =)		Breakdown products	
4-hydroxy- sulfamethoxazole [a] (4-OH-SMX)		Sulfanilamide (SFA)	
N^4-hydroxy- sulfamethoxazole (*N*-OH-SMX)		3-amino-5-methylisoxazole (3A5MI)	
4-nitroso-sulfamethoxazole (NO-SMX)		Sulfanilic acid (SA)	
4-nitro-sulfamethoxazole (NO_2-SMX)		Benzensulfonamide (BSA)	
N^4-acetyl-sulfamethoxazole (acetyl-SMX)		Aniline (AN)	
N^4-hydroxy-acetyl-sulfamethoxazole [a] (OH-acetyl-SMX)			

2. Materials & Methods

2.1. Chemicals

3-Amino-5-methylisoxazole (CAS 1072-67-9), sulfanilic acid (CAS 121-57-3), sulfanilamide (CAS 63-74-1), benzenesulfonamide (CAS 98-10-2), aniline (CAS 62-53-3) and sulfamethoxazole (723-46-6) were purchased from Sigma-Aldrich (Oakville, ON, Canada). N^4-acetyl-sulfamethoxazole (CAS 21312-10-7), 4-nitroso-sulfamethoxazole (CAS 131549-85-4, purity >90%), N^4-hydroxy-sulfamethoxazole (CAS 114438-33-4) and 4-nitro-sulfamethoxazole (29699-89-6) were purchased from Toronto Chemicals (Toronto, ON, Canada). 4-NO-SMX was stored at -20°C. Freeze-dried luminescent bacteria (Acute Reagent, 855-637-6426) were purchased from Modern Water (New Castle, DE, USA) and stored at -20°C until use.

2.2 Synthesis of OH-acetyl-SMX, 4-NO_2-SMX and 4-OH-SMX

Synthesis of 4-OH-SMX was previously reported in literature (Vidyasagar et al., 1991). However, reproduction of the synthesis of 4-OH-SMX using the reported procedure did not yield satisfying results. Therefore, the desired product was synthesized in two steps via the previously unknown 4-methoxy-*N*-(5-methylisoxazol-3-yl)benzenesulfonamide and following

cleavage of the methoxy group with boron tribromide. The detailed procedure, corresponding analytical nuclear magnetic resonance (NMR) and mass spectrometry data is given in S1 Supporting information.

4-Hydroxy-N-(5-methylisoxazol-3-yl)benzenesulfonamide (4-OH-SMX)

To a solution of 4-methoxy-N-(5-methylisoxazol-3-yl)benzenesulfonamide (800 mg, 2.98 mmol, 1 equiv.) in dry dichloromethane (15 mL) at −78 °C boron tribromide solution (1 M in dichloromethane, 8.95 mL, 8.95 mmol, 3 equiv.) was added dropwise under inert atmosphere with stirring. After 30 minutes, the reaction mixture was warmed up to room temperature and stirred for further 5 hours. It was then quenched and neutralized with saturated $NaHCO_3$ solution and extracted with ethyl acetate (3 × 30 mL). The organic phase was dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. Flash chromatography (silica gel, cyclohexane/ethyl acetate 1/1 v/v) of the crude product afforded 310 mg (41% yield) of the title compound as a yellow solid.

***N*^{4}-Hydroxyacetylsulfamethoxazole (OH-acetyl-SMX)**

Sulfamethoxazole (700 mg; 2.76 mmol; 1 equiv.) was dissolved in dry tetrahydrofuran (14 mL) under inert atmosphere. Triethylamine (1.11 mL; 8.02 mmol; 2.9 equiv.) and acetoxyacetyl chloride (1.02 g; 7.46 mmol; 2.7 equiv.) were added and the reaction mixture was stirred at room temperature for 16 hours. It was then concentrated under reduced pressure. Subsequently, 1.5 N aqueous sodium hydroxide solution (20 mL) and methanol (20 mL) were added to the resulting residue, and the mixture was again stirred at room temperature for 12 hours. The reaction mixture was concentrated under reduced pressure and the residue was then dissolved in water. The solution was neutralized with 1 N aqueous HCl and then extracted with ethyl acetate (3 × 30 mL). The organic layer was washed with saturated brine, dried over anhydrous sodium sulfate and filtered. The filtrate was concentrated under reduced pressure and the crude product purified by column chromatography (silica gel, cyclohexane/ethyl acetate 1/2 – 0/1) to afford 670 mg (78% yield) of the title compound as yellowish crystals.

2.3. Bioassays

Short-term tests using LI endpoints such as 15 min or 30 min usually fall short of the delayed bacteriostatic effects of antibiotics and therefore, can significantly underestimate the ecotoxicological effects (Backhaus and Grimme, 1999; Kümmerer, 2009). For that reason, long-term bioassays over 24 h were applied here, providing a growth medium with carbon

and nutrient sources (Froehner et al., 2002) to sustain the bacteria and their luminescence light emission over this extended test duration. This test approach also allows to track bacterial growth by optical density measurements in addition to luminescence light emission (Menz et al., 2013). Apart from that, experiments were carried out according to ISO 11348-3 and repeated in independent experiments for each compound. The initial pH was 6.8 and no further adjustments were done. Luminescence and optical density (OD) were measured after 30 min (endpoint most commonly applied) and 24 h, respectively. Relative luminescence light emission was measured by a Microtox™ M500 Analyzer (Modern Water, New Castle, DE, USA; formerly SDI). Relative absorbance was measured by a HACH photometer at λ = 600 nm. Pipette tips and glassware used for stock solutions and substrate were autoclaved for 20 min at 121°C before usage to avoid microbial contamination. A detailed description of the procedure, the composition of the substrates and of test validation experiments regarding the interaction of SMX TPs with the growth substrate, possible light absorption, substrate competition and comparison to ISO 11348-3 can be found in S2 (Supporting Information).

The Hill equation was used to fit the monotonic dose-response relationships obtained from the bioassays by minimizing chi^2 between modeled and measured data using the OriginPro 8.5 software (Originlab Corporation, USA):

(1)

where I = inhibition, I_0 = the minimum inhibition (set to I_0 = 0 %), I_{max} = maximum inhibition (set to I_0 = 100 %), c = the toxicant concentration in [µmol L^{-1}], EC_{50} = effect concentration at I = 50% and n = Hill coefficient.

2.4 Determination of pK_{a2}

Acidic dissociation constants for the N^1 amino group (pK_{a2}) of the two synthesized metabolites 4-OH-SMX and OH-acetyl-SMX as well as of *N*-OH-SMX (not available in literature) were determined by measuring absorbance in titration experiments over a pH range from 2 to 10 using HCl and NaOH (c = 0.05 mol L^{-1}) in tap water. An automatic titrator (SI Analytics) was used to adjust the pH and absorbance measurements were carried out using a photometer (Cary 50, Varian) at λ = 235 nm for 4-OH-SMX and OH-acetyl-SMX, and λ = 245 nm for *N*-OH-SMX. Wavelengths were selected based on UV/Vis spectra (200 nm to 400 nm) at pH = 3 and pH = 11. Experimental data were fitted with the Boltzmann equation. For the pH range typically prevailing in aquatic environments, only the pK_{a2} is relevant.

Experimental pK_{a2} values for SMX and the other TPs were taken from literature and theoretical pK_{a2} values for all compounds were also calculated for comparison using *Marvin Sketch* (ChemAxon, Hungary) (Table S1, Supporting information).

3. Results & Discussion

3.1 Growth and luminescence test validation

Compared to the ISO test conditions using only a salty test medium, the approach applied here used additional carbon and nutrient sources to ensure luminescence light emission over a longer period and to allow *v. fischeri* to grow. Both are inherently linked since around 10 % of the metabolic energy is converted into luminescence light production (Klopman and Stuart, 2003) and in this way related to the cellular respiration. A series of validation experiments with SMX and SFA, as representatives for ionized and neutral compounds at test pH, showed that the growth medium does not interfere with the test results. Testing of SMX with the growth medium provided a dose-response relationship matching the one obtained using ISO 11348-3 (Figure S3). The detailed data and further results of the validation experiments are given in S3 Supporting Information).

3.2 Dose-response relationships of SMX TPs

3.2.1 Luminescence inhibition (LI)

All breakdown products were more than two to three orders of magnitude less active than SMX resulting in EC_{50} values between 900 µmol L^{-1} and 9709 µmol L^{-1} corresponding to the upper mg L^{-1} mass concentration range (Table 2). 3A5MI, which was reported to be a dead-end metabolite of SMX (Müller et al., 2013) and thus, may accumulate in the environment, showed LI_{24h} at EC_{50} = 2.7 mmol L^{-1} (264.9 mg L^{-1}), while no effect was observable after 30 min. The ratio of both LI endpoints, termed here as acute-to-chronic-ratio (ACR), was between 1 and 3 for the breakdown products, i.e. their EC_{50} at both exposure times changed only marginally (Figure 2a,b).

The picture is substantially different for SMX and its derivatives (Table 2). As can be seen in Figure 2c,d, results for LI after 30 min and 24 h differed considerably for both SMX and its derivative TPs. The ACRs show that the EC_{50} for these compounds were 14 to 79 times lower after 24 h than after 30 min. This indicates their bacteriostatic mechanism of action, whose effect only becomes apparent after many microbial reproduction cycles. These results are in agreement the findings previously reported for other parent antibiotics by

Backhaus et al. (1997). No ACR value could be derived for OH-acetyl-SMX since its LI EC_{50} values were not reached within the exposure time.

The LI_{24h} EC_{50} of SMX was at 7 µmol L^{-1} (1.7 mg L^{-1}). Expressing the toxicity of the derivatives relative to SMX as toxicity equivalents (TE_{SMX}), it can be seen that NO_2-SMX and NO-SMX are similarly toxic as the parent, followed by 4-OH- and *N*-OH-SMX, while the two acetylated derivatives seem to exhibit very low antibacterial activity after 24h. After 30 min, NO-SMX, 4-OH-SMX and NO_2-SMX even acted 1.2 to 3.3 times more toxic upon luminescence emission, which can however be attributed to an immediate interference with the cellular respiration but no bacteriostatic effect due to the short exposure time.

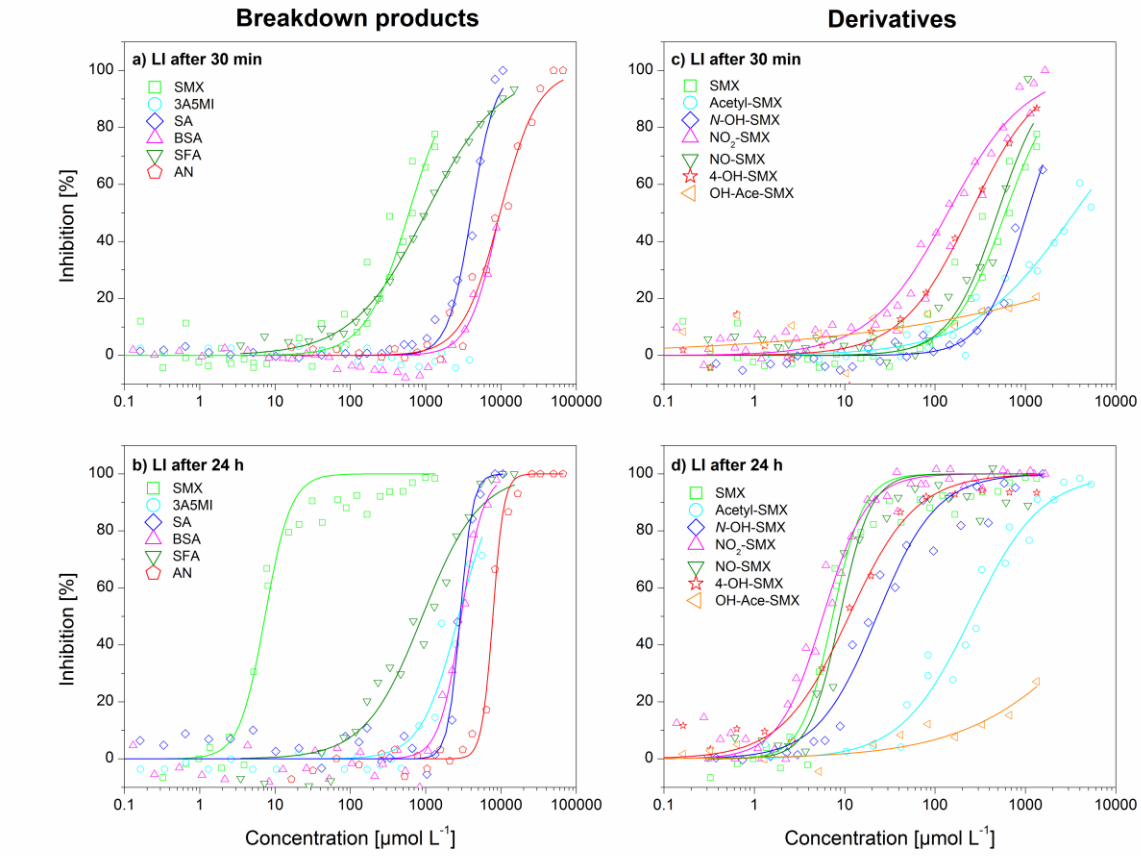


Figure 2a-d. Dose-response relationships of SMX TPs and *v. fischeri*; SMX is shown in all four graphs for comparison; data were fitted with the Hill equation; n = 15 to 39; all r^2 were >0.91 except for OH-Ace-SMX (0.24, 0.62) indicating that the proportion of variation accounted for by the Hill model compared to the *null model* (mean of the y-values) is smaller than for the other compounds; please note the different x-axis scales for breakdown products and derivatives.

3.2.2 Growth inhibition (GI)

Growth was generally less affected by the tested compounds than LI as indicated by the clearly higher GI EC_{50} values. All breakdown products show considerably lower toxicity by 1

to 2 orders of magnitude as compared to SMX (Table 2; Figure S5 and S6 Supporting Information) indicating again the loss of the bacteriostatic mechanism of action. 3A5MI did not showed any GI effects for the tested concentration range of up to 5.6 mmol L^{-1} (549 mg L^{-1}). Further, the two acetylated TPs are rather inactive towards GI with EC_{50} of 7.6 mmol L^{-1} and 5.6 mmol L^{-1}, respectively, which corresponds to less than 10% of the residual activity of SMX. On the contrary, the 4-OH- and the NO_2-derivative are 5.7 and 21.4 times more toxic relative to SMX. Also, *N*-OH-SMX exhibited a GI effect of around 80% that of SMX. For this endpoint, the order of toxicity relative to SMX among the derivatives has changed to (listed by their R^1) NO_2- > 4-OH- > NH_2- (parent) > *N*-OH- > *N*-acetyl-, N-OH-acetyl- > NO-. NO-SMX exhibited surprisingly low GI when compared to its LI_{30min} and LI_{24h} values. It is known that NO-SMX is reactive and very unstable in solutions and therefore, the high GI EC_{50} values are likely to be due to decomposition or further transformation of the compound (Naisbitt et al. 1996). The different order of toxicity observed with GI implies that LI cannot be used as a proxy in order to predict the GI with sufficient certainty, also when ignoring the results of NO-SMX. Moreover, no significant correlation could be found between the ACR and GI. Although luminescence is clearly adversely affected by the TPs through GI, it is not the sole mechanism as shown by the EC_{50} after 30min, during which no detectable growth and GI takes place.

Table 2. EC_{50} values, sulfamethoxazole toxicity equivalents (TE_{SMX}) and acute-to-chronic ratios (ACR) of sulfamethoxazole and the selected transformation products; the TPs are ordered by their TE_{SMX} of LI_{24h}; ACR = ratio of EC_{50} LI_{30min} and EC_{50} LI_{24h}.

	EC_{50} LI_{30min} *[µmol L^{-1}]*	**EC_{50} LI_{24h}** *[µmol L^{-1}]*	**EC_{50} GI_{24h}** *[µmol L^{-1}]*	**TE_{SMX} LI_{30}** *[-]*	**TE_{SMX} LI_{24}** *[-]*	**TE_{SMX} GI_{24}** *[-]*	**ACR** *[-]*
SMX	551	7	599	1	1	1	79
NO_2-SMX	168	6	28	3.3	1.2	21.4	28
NO-SMX	464	9	19822	1.2	0.8	<0.05	52
4-OH-SMX	245	11	106	2.2	0.6	5.7	22
N-OH-SMX	1044	23	788	0.5	0.3	0.8	45
N-Acetyl-SMX	3466	252	7567	0.2	<0.05	0.1	14
N-OH-Acetyl-SMX	>1333	>1333	5590	<0.4	<0.05	0.1	-
SFA	1006	900	23813	0.65	0.01	0.06	1
SA	4057	2867	6854	0.16	<0.01	0.21	1
BSA	9534	2966	18687	0.07	<0.01	0.08	3
AN	9709	7780	24805	0.07	<0.01	0.06	1
3A5MI	>5596	2731	>5596	<0.1	<0.01	<0.1	>2

3.3 Mixture Toxicity

Given the results of the experiments with the *para*-modified TPs compounds and their presumably same mechanism of action, it was expected that the effect of a mixture of SMX derivatives can be described by the concept of concentration addition (CA) (Backhaus and Faust, 2012), where the effect of a mixture is the sum of its effects at given individual compound concentrations. The CA approach is theoretically expressed as:

$$\left(\sum \quad \text{—}\right) \tag{2}$$

where $EC_{x,mix}$ is the effect concentration where the x % is affected by the mixture, n is the number of compounds in the mixture, p_i = proportion of each single compound concentration to the total concentration and EC_{xi} = is the effect concentration of the single compound at a given affected fraction of x %.

It was assumed that the tested compounds did not interact with each other on a molecular level nor with regard to their toxicokinetics. As can be seen from Figure 3, the CA concept was well suited to describe the effect of an equimolar mixture of five compounds (SMX, NO_2-SMX, NO-SMX, *N*-OH-SMX and *N*-acetyl-SMX) for the LI and to a lesser extent for GI, where the model slightly overestimates the experimental values. This might be due to the low stability of NO-SMX, which may have been transformed (Naisbitt et al., 1996) and thus, was possibly no longer capable of enzyme binding. Nonetheless, these results indicate that the derivative transformation products of SMX can contribute to the total antibacterial effect and therefore, need to be considered in addition to the parent compound. Taking one step further, it is consistent to assume that this holds valid for any sulfonamide present in a water body due to their common molecular structure and mechanism of action (cf. section 4).

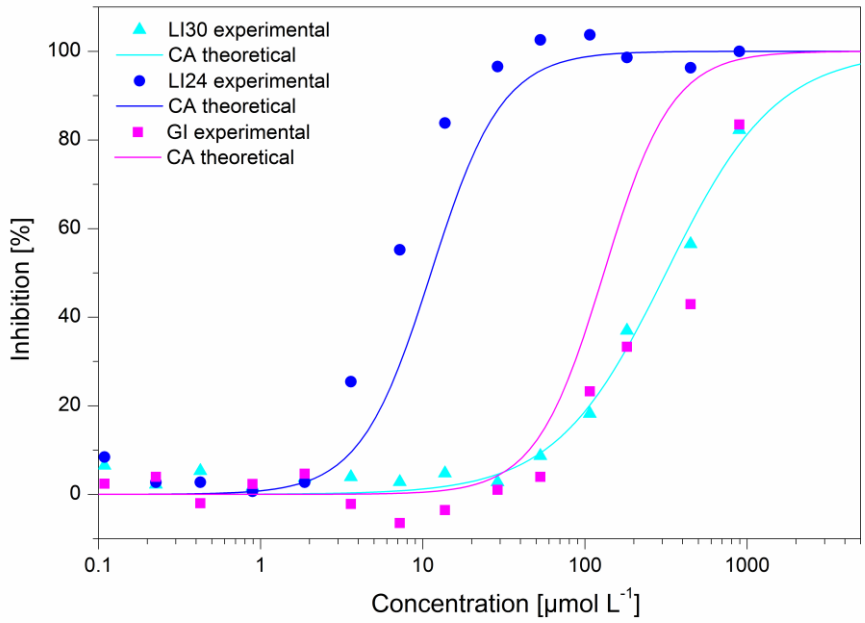


Figure 3. Experimental mixture toxicity of SMX, NO_2-SMX, NO-SMX, *N*-OH-SMX and *N*-acetyl-SMX as well as their theoretical mixture toxicity according to the concentration addition concept (CA, Eq. 2); starting concentration c = 0.8 mmol L^{-1} per compound in mixture.

3.4 Relationship between antibacterial activity and *para*-transformations

Results showed that the toxicity of the breakdown products SFA and BSA is considerably lower as compared to the parent SMX, which both feature the benzenesulfonamide moiety. Although the sulfanilamide building block is the toxicophore isosteric to *p*ABA, the low toxicity of the latter two compounds can be attributed to their presence as a neutral species at pH 6.8 with pK_a of 10.58 and 10.10, respectively. Further, BSA lacks the primary amino group at the *para*-position, which is supposed to be paramount to interference with the folic acid synthesis by binding to 6-methylpterin. Further details about the interaction of sulfonamides with the microbial folic acid pathway can be found elsewhere (Richter et al., 2013). In contrast, *p*ABA exhibits a pK_{a2} of around 4.7 and thus, is present in its negatively charged species to 99% at pH 6.8. This agrees with the findings by Hansch (2003), who concluded from quantitative-structure-activity-relationships (QSAR) studies on *E. coli* that the ionized amide group of the sulfonamide moiety promotes the biological effects.

All breakdown products including the latter two however still provoke GI and LI at high doses > 0.9 mmol L^{-1}. This and the fact that there is no delayed effect on LI as indicated by the ACRs, suggests another mechanism of action, most likely of an unspecific narcosis type. In this regard, the LI_{30min} and LI_{24h} of the four breakdown products originating from the

4-aminobenzenesulfonamide moiety (SA, SFA, AN and BSA) also showed a positive correlation with their log K_{ow} (Table S1, Supporting Information).

In view of the results of single substance and mixture toxicity experiments, the SMX derivatives are supposed to act via the same mechanism of action as the parent SMX. Together they form a homologous series with different substitutes in the *p*-position. QSAR have been established for parent sulfonamide homologs that unraveled their mechanisms of actions (Seydel, 1981). However, in these studies, only the R^2 moiety was modified, while the *p*-amino group remained, since up to now this group has been considered relevant for their mechanism of action. For instance, Nouws et al. (1985) concluded from the antibiotic inactivity of N^4-acetyl-sulfonamides on *E. coli* that a free *p*-aminophenyl group is required. To our knowledge, the results of the present study showed for the first time that SMX transformed at the *para*-position can still exhibit and even increase its ecotoxicity. To explain this phenomenon, two mechanisms of action come into consideration: a) competitive inhibition of the active site of the key enzyme dihydropteroate synthase (DHPS) for folic acid synthesis and/or ii) incorporation of the TP in lieu of *p*ABA, which leads to inactive products.

By analogy to the parent sulfonamides, we first hypothesized different degrees of ionization of the TP and changes in the electron distribution (expressed as their pK_{a2} values) at the given pH to be responsible for the measured effects, which would indicate competitive protein binding. In order to test this assumption, the pK_{a2} values of the two synthesized metabolites as well as *N*-OH-SMX needed to be experimentally determined since no data were available in literature (Table 3).

Table 3. Experimental pK_{a2} values of 4-hydroxy-sulfamethoxazole, N^4-hydroxy-acetyl-sulfamethoxazole and N^4-hydroxy-sulfamethoxazole; data fitted with Boltzmann equation; see Figure S7 (Supporting Information).

Compound	pK_{a2}	± Confidence bands (95%)	*n*	r^2
4-OH-SMX	4.89	± 0.09	24	0.97
OH-Acetyl-SMX	5.43	± 0.05	24	0.99
N-OH-SMX	4.51	± 0.04	17	0.99

Plotting the experimental pK_{a2} values versus the GI EC_{50} endpoints showed increasing toxicity with increasing pK_{a2} values, thus decreasing degree of ionization for NO-, *N*-acetyl-, *N*-OH-acetyl-SMX and SMX itself (Figure 4). These data match well the data reported in literature for parent sulfonamides, which indicated an optimum at around pK_{a2} = 6.5, before and after which the activity decreases (Bell and Roblin, 1942; Seydel, 1981). The data of this

study presents only the lower branch of the optimum, since all SMX-TPs exhibit pK_{a2} < 5.9. However, three derivatives are significantly more growth inhibiting than expected, namely NO_2-, *N*-OH and 4-OH-SMX. It should be noted that the differences of these three compounds are only observable when using the experimental pK_{a2} values which were up to two pK_a units lower as the theoretical ones.

The substituents of these three derivatives exert a negative inductive effect, which facilitates the release of the proton of the amide group, and thus may result in a stronger affinity to bind to DHPS. However, these are also stronger nucleophiles than the common *p*-amino group of the sulfonamides, which may lead to an increased amount of faulty products by the replacement of *p*ABA by covalent binding. Yun et al. (2012) showed that *p*-hydroxybenzoic acid (*p*HBA), which is structurally similar to 4-OH-SMX, bound to the active site of the responsible enzyme. Nevertheless, the exact mechanism cannot be elucidated by the data of this study.

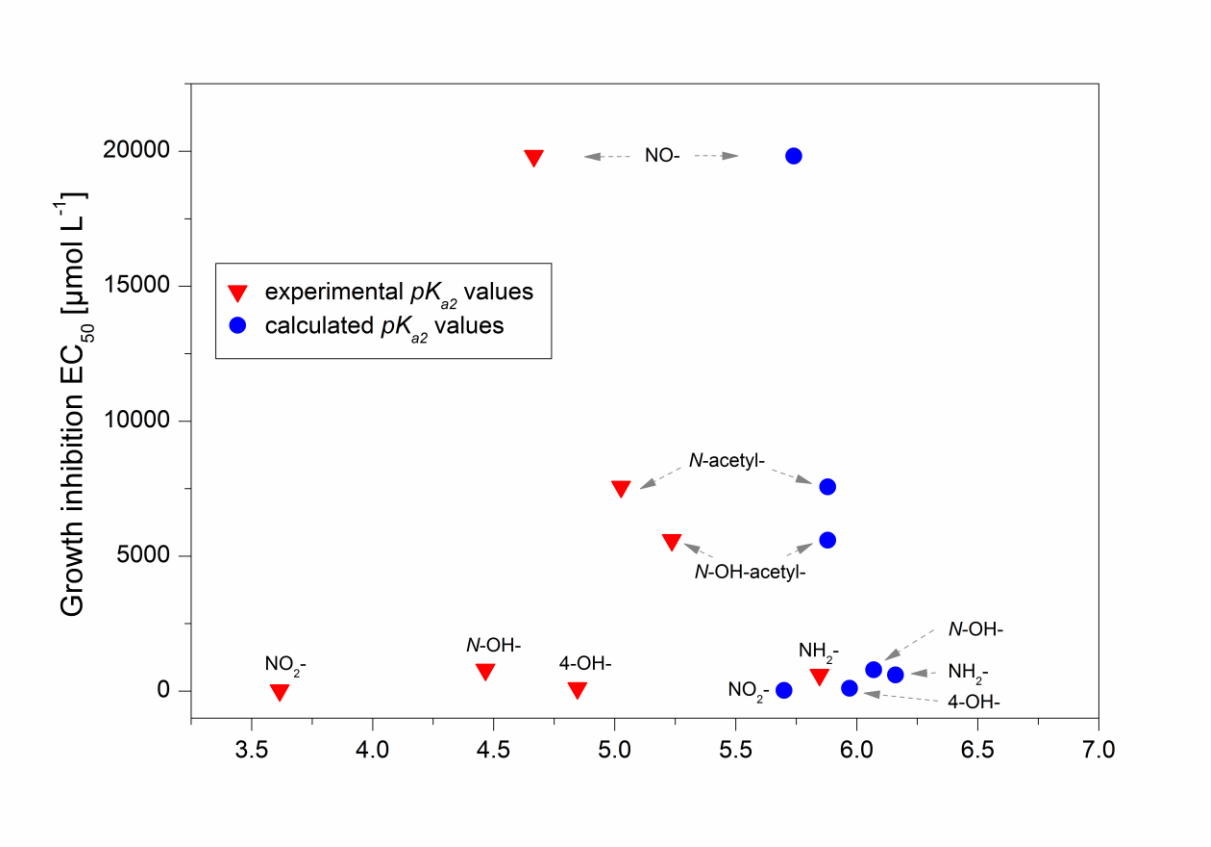


Figure 4. Relationship between the acidic constant pK_{a2} and GI EC_{50} values plotted for theoretical and experimental pK_{a2} values; experimental values taken from literature as well as theoretical values are listed in Table S1.

4 General relevance of *para*-modified sulfonamide TPs

The ecotoxicological screening using *v. fischeri* presented in this study can certainly be only a small contribution to a holistic environmental risk assessment. It requires testing for other bacterial species and higher trophic levels as well as data about the occurrence and persistence in wastewater and surface water of these compounds, which was out of scope of this study. However, based on this screening and considering that SMX is a representative example of sulfonamide antibiotics, inevitably the question arises about the importance considering *para*-TPs formed from other homologous sulfonamides.

All TP SMX derivatives considered in this study underwent transformation at the *para* binding site, a site that is present in almost all sulfonamide antibiotics with only a very few exceptions. Accepting the hypothesis that the ionization of the secondary amine largely promotes biological effects, transformation reactions of sulfonamide homologs with electron withdrawing moieties such as nitro or hydroxyl groups are generally suggested to be ecotoxicologically relevant. The *para*-TPs still might act as a *p*ABA substitute, which leads to inactive products, i.e. that these TPs exhibit some relevant ecotoxicity of the parent compound towards *v.fischeri* and most likely also towards other bacterial species.

As shown in this study, the R_2 moiety alone being released from sulfonamide bonding cleavage can be assumed to be of minor ecotoxicological relevance. Consequently, focus has to be put on the derivatives presumably bearing ecotoxicological potential.

Understanding the total antibiotic stress as the sum of the effects of all compounds with this mechanism of action in an aquatic system, as the concept of concentration addition suggests, a profound ecological risk assessment principally needs to consider for all sulfonamides and derivative TPs present. Realistically, their cumulative amount acts as the key factor for possible microbial antibiotic resistance. Coping with this issue, one option consists in a combined approach by quantifying the total impact by sum-parameters via biomarkers (Richter et al., 2013) or sulfonamide ELISA as well as prioritization and determination of single relevant TPs.

Bearing in mind that extrapolation from *in vitro* to *in vivo* has its restrictions and considering that it seems unlikely that EC_{50} values of GI might be observed for individual compounds or mixtures in the aquatic environment (lowest GI values were in the mg L^{-1} range), the risk of GI posed by these compounds seems limited. Moreover, knowing that *v. fischeri* is a marine species, testing of further species native to surface water habitats is advisable. However, recent research gave insight about the sulfonamide resistance mechanisms suggesting that the latter is associated with the R^2 moieties in the sulfa drug,

which have no equivalent in *p*ABA, and are located outside the DHPS substrate envelope where mutations may impede sulfa drug binding (Yun et al., 2012). In this regard, the derivative TPs should be of equal interest as the parent compound as they may promote antibiotic resistance in the same way. To our knowledge, it was observed for the first time that sulfonamides act via their bacteriostatic mode of action although they did not feature the free primary amino group. As for the breakdown products, SA, SFA, AN and BSA, which are common to all sulfonamides as well as the R^2 moiety 3A5MI, they all exhibited low GI and LI.

These results provided insights on the potential environmental impact of *para*-modified TPs of SMX, but still relatively is known about their occurrence, persistence and origin. Further monitoring is needed to ensure that the risk of sulfa drugs and their TPs is not underestimated by only focusing on the parent compounds.

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