Supporting Information

for

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

Marius Majewsky[a], Danny Wagner[b], Markus Delay[a],
Stefan Bräse[b,c], Viviane Yargeau[d], Harald Horn[a]

[a] *Karlsruhe Institute of Technology (KIT), Engler-Bunte-Institut, Chair of Water Chemistry and Water Technology, Engler-Bunte-Ring 1, 76131 Karlsruhe, Germany*

[b] *Karlsruhe Institute of Technology (KIT), Institute of Organic Chemistry, Fritz-Haber-Weg 6, 76131 Karlsruhe, Germany*

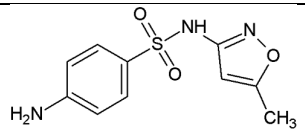
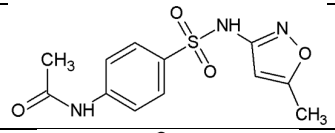
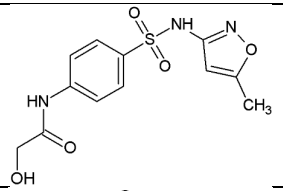
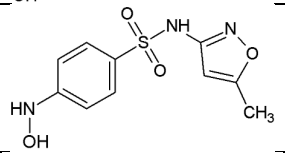
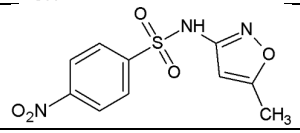
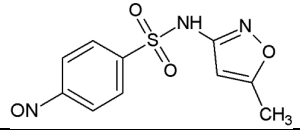
[c] *Karlsruhe Institute of Technology (KIT), Institute of Toxicology and Genetics (ITG), Hermann-von-Helmholtz-Platz 1, 76344 Eggenstein-Leopoldshafen, Germany*

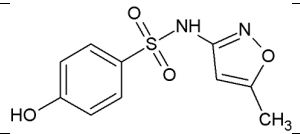
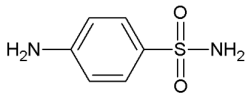
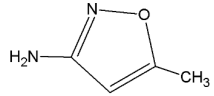
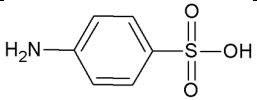
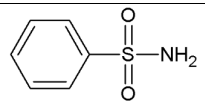
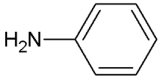
[d] *Department of Chemical Engineering, McGill University, 3610 University St., Montreal, QC, J3N 1V3, Canada*

- 12 pages
- 7 figures (S1 to S7)
- 1 table (S1)

*Corresponding author: marius.majewsky@partner.kit.edu; Tel: + 49 721 608 - 4 2788.

Table S1. Molecular structures, physico-chemical properties and formation processes of sulfamethoxazole and the 11 tested transformation products reported in literature; [a] computed using MarvinSketch; [b] SRC PhysProp database; [15] [c] Lin et al., 1997; [16] [d] this study; [e] Bonvin et al., 2012; [6] *exp.* = experimental.

Compound	Structure	pK_{a2} *exp.*	pK_{a2} [a]	Ionized N^4 amino group at pH 6.8[a] [%]	log *D* at pH 6.8[a]	log K_{ow} *exp.*[b]	log K_{ow} [a]	Potential formation process
Sulfamethoxazole (SMX)		5.89[c]	6.16	81.3	0.24	0.89	0.79	-
N^4-acetyl-sulfamethoxazole (acetyl-SMX)		5.07[e]	5.88	89.3	0.18	1.21	0.86	Human metabolism[1] Microbial transformation in water/sediment[2]
N^4-hydroxy-acetyl-sulfamethoxazole (OH-acetyl-SMX)		5.28[d]	5.88	89.3	-0.64	-	0.04	Microbial transformation in activated sludge bacteria (hypothesized)[3]
N^4-hydroxy-sulfamethoxazole (*N*-OH-SMX)		4.51[d]	6.07	84.4	0.53	-	1.13	Human metabolism[1]
4-nitro-sulfamethoxazole (NO_2-SMX)		3.66[e]	5.7	92.7	0.81	-	1.56	Microbially mediated abiotic formation during wastewater treatment[4,5] Human metabolism[6]
4-nitroso-sulfamethoxazole (NO-SMX)		4.71[e]	5.74	92.0	0.96	-	1.70	Human metabolism[6]

4-hydroxy-sulfamethoxazole (4-OH-SMX)		4.89[d]	5.97	86.0	0.67	-	1.32	Microbial transformation by activated sludge bacteria (hypothesized)[3,7]
Sulfanilamide (SFA)		10.58[b]	10.99	0.01	-0.25	-0.62	-0.25	Photolysis[8]
3-amino-5-methylisoxazole (3A5MI)		-	-	-	0.3	-	0.3	Microbial transformation[7,9] Photolysis[6,10,11]
Sulfanilic acid (SA)		-	-	-	-2.04	-2.16	0.33	Photolysis[6,8,10,11]
Benzensulfonamide (BSA)		10.10[b]	10.42	0.04	0.58	0.31	0.58	Microbial transformation from high molecular weight sulfonamides (assumed)[12]
Aniline (AN)		-	-	-	1.14	0.9	1.14	Photolysis[1,6,8,10]

S1 Synthesis and Analytical Data

Synthesis of 4-Methoxy-N-(5-methylisoxazol-3-yl)benzenesulfonamide as an intermediate

3-Amino-5-methylisoxazole (1.00 g, 10.19 mmol, 1 equiv.) was dissolved in pyridine (11 mL) and cooled to 0 °C. Subsequently, *p*-methoxybenzenesulfonyl chloride (2.53 g, 12.2 mmol, 1.2 equiv.) was added in portions over 20 minutes. The reaction mixture was warmed up to room temperature and stirred overnight. Water (50 mL) was added and the mixture was stirred for further 20 min. The precipitate was filtered and washed with water. The crude product was then dissolved in 2 N NaOH (30 mL), filtered to remove insoluble by-products, and precipitated from the filtrate with 1 N HCl. The resulting precipitate was again filtered, washed with water and dried to afford 2.36 g (86% yield) of the title compound as a white powder.

Nuclear Magnetic Resonance (NMR), Mass Spectrometry and High Resolution Mass Spectrometry (MS; HRMS), and Infrared Spectroscopy (IR) Data

4-Methoxy-N-(5-methylisoxazol-3-yl)benzenesulfonamide (intermediate)

NMR. ^{1}H NMR (400 MHz, [D$_6$]-DMSO): δ = 2.28 (s, 3 H, C*H*$_3$), 3.81 (s, 3 H, OC*H*$_3$), 6.13 (s, 1 H, $_{isox.}$), 7.11 (d, 2 H, H $_{ar.}$, $^{3}J_{HH}$ = 8.9 Hz), 7.78 (d, 2 H, H $_{ar.}$, $^{3}J_{HH}$ = 8.9 Hz), 11.29 (s, 1 H, N*H*) − ^{13}C NMR (400 MHz, [D$_6$]-DMSO): δ = 12.0 (+, *C*H$_3$ isox.), 55.7 (+, O*C*H$_3$), 95.3 (+, 4'-*C*H$_{isox}$), 114.5 (+, 3,5-*C*H), 129.0 (+, 2,6-*C*H), 131.0 (C$_q$, 1-*C*$_{Ar}$), 157.6 (C$_q$,3'-*C* $_{isox}$), 162.8 (C$_{q,}$ 4-*C*$_{Ar}$), 170.2 (C$_{q,}$5'-*C* $_{isox}$). ppm.

MS and HRMS. MS (FAB, 3-NBA), *m/z* (%): 269 (100) [M+H$^+$]. HRMS ($C_{11}H_{13}O_4N_2S$): calcd. 269.0591; found 269.0589

IR. IR (ATR): $\tilde{\upsilon}$ (cm^{-1}) = 3094 (vw), 2981 (vw), 2843 (vw), 1609 (w), 1593 (w), 1496 (w), 1472 (w), 1438 (w), 1393 (w), 1338 (w), 1315 (w), 1260 (m), 1182 (w), 1163 (m), 1114 (w), 1090 (w), 1031 (w), 1007 (w), 931 (w), 897 (w), 843 (w), 796 (m), 712 (w), 688 (w), 667 (m), 635 (w), 570 (m), 551 (s), 489 (w).

Elemental analysis. Calcd. for $C_{11}H_{12}N_2O_4S$: C 49.24, H 4.51, N 10.44, S 11.95; found C 49.26, H 4.52, N 10.26, S 11.98.

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

NMR. ^{1}H NMR (400 MHz, [D$_6$]-DMSO): δ = 2.29 (s, 3 H, C*H*$_3$), 6.11 (s, 1 H, $_{isox.}$), 6.90 (d, 2 H, H $_{ar.}$, $^{3}J_{HH}$ = 8.8 Hz), 7.67 (d, 2 H, H $_{ar.}$, $^{3}J_{HH}$ = 8.7 Hz), 10.56 (s, 1 H, O*H*), 11.19 (s, 1 H, N*H*). – ^{13}C NMR (400 MHz, [D$_6$]-DMSO): δ = 12.1 (+, *C*H$_{3\ isox.}$), 95.4 (+, 4'-*C*H$_{isox}$), 115.7 (+, 3,5-*C*H), 129.2 (+, 2,6-*C*H), 129.3 (C$_q$, 1-*C*$_{Ar}$), 157.7 (C$_{q,}$3'-*C* $_{isox}$), 161.7 (C$_{q,}$ 4-*C*$_{Ar}$), 170.2 (C$_{q,}$5'-*C*$_{isox}$). ppm.

MS and HRMS. MS (FAB, 3-NBA), *m/z* (%): 255 (100) [M+H$^+$]. – HRMS ($C_{10}H_{11}O_4N_2S$): calcd. 255.0434; found 255.0433

IR. IR (ATR): $\tilde{\upsilon}$ (cm^{-1}) = 3246 (w), 1600 (w), 1584 (w), 1469 (w), 1435 (w), 1388 (w), 1374 (w), 1330 (w), 1287 (w), 1259 (w), 1218 (w), 1175 (w), 1160 (m), 1103 (w), 1089 (w), 1029 (w), 1007 (w), 927 (w), 878 (w), 837 (w), 820 (w), 789 (w), 686 (m), 634 (w), 605 (w), 565 (w), 544 (m), 446 (vw).

Elemental analysis. Calcd. for $C_{10}H_{10}N_2O_4S$: C 47.24, H 3.96, N 11.02, S 12.61; found C 46.52, H 3.97, N 10.36, S 12.36.

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

NMR. ^{1}H NMR (400 MHz, [D$_6$]-DMSO): δ = 2.29 (s, 3 H, C*H*$_3$), 4.03 (s, 2 H, COC*H*$_2$OH), 5.74 (br s, 1 H, N*H*CO) 6.14 (s, 1 H, isox.), 7.79 (d, 2 H, H$_{aromat.}$, $^{3}J_{HH}$ = 8.6 Hz), 7.91 (d, 2 H, H$_{aromat.}$, $^{3}J_{HH}$ = 8.6 Hz), 10.12 (s, 1 H, SO$_2$N*H*), 11.35 (s, 1H, CH$_2$O*H*) – ^{13}C NMR (400 MHz, [D$_6$]-DMSO): δ = 12.0 (+, *C*H$_{3\ isox.}$), 61.9 (–, *C*H$_2$OH), 95.3 (+, 4'-*C*H), 119.4 (+, 3,5-CH), 127.9 (+, 2,6-CH), 133.3 (C$_q$, 1-*C*$_{Ar}$), 142.8 (C$_q$, 4-*C*$_{ar.}$), 157.6 (C$_{q,}$3'-*C* $_{isox}$), 170.3 (C$_{q,}$ 5'-*C*$_{isox.}$), 171.7 (C$_{q,}$ NH*C*O). ppm.

MS and HRMS. MS (FAB, 3-NBA), *m/z* (%): 312 (28) [M+H$^+$], 307.2 (22), 289.1 (15), 154 (100). – HRMS ($C_{12}H_{14}O_5N_3S$): calc. 312.0649; found 312.0650.

IR. IR (ATR): $\tilde{\upsilon}$ (cm^{-1}) = 3363 (vw), 3289 (w), 3216 (w), 2924 (vw), 1662 (w), 1613 (w), 1594 (w), 1543 (m), 1510 (w), 1461 (w), 1390 (w), 1324 (w), 1267 (w), 1210 (w), 1187 (w), 1162 (m), 1077 (m), 1029 (w), 1008 (w), 986 (w), 925 (w), 899 (w), 855 (w), 833 (w), 786 (w), 727 (w), 619 (m), 566 (m).

Elemental analysis. Calcd. for $C_{12}H_{13}\ N_3O_5S$: C 46.30, H 4.21, N 13.50, S 10.30; found C 46.70, H 4.36, N 13.13, S 10.11.

S2 Substrate composition and test validation experiments

***Growth medium*.** Seawater complete medium (SWC) was prepared according to ISO 11348-3 with NaCl (20 g L^{-1}), $MgCl_2 \cdot 6\ H_2O$ (2 g L^{-1}) and KCl (0.3 g L^{-1}) in double distilled water for use in validation tests between the ISO method and the bioassay with growth substrate. Since the luminescent light emission of *V. fischeri* rapidly decreases within a few hours, additional carbon and nutrient sources have to be added in order to maintain luminescent light emission[13] and facilitate bacterial growth, which can be simultaneously tracked by optical density measurements.[14] This additional growth medium was prepared using the salts of the SWC at the same concentrations as well as yeast extract (5 g L^{-1}), trypton (5 g L^{-1}) and glycerol (3 g L^{-1}). All stock solutions of SMX and the TPs were prepared in growth medium to avoid dilution effects when adding to the samples. Same amounts of pure growth medium were added to the controls ensuring identical volumes and nutrient contents.

Test Procedure. 1 mL of growth substrate was added to each Microtox™ glass tubes and acclimatized to 15°C for approximately 1 h. Dilution series of the target compounds were carried out separately by 1:1 dilutions in growth substrate from the lower µmol L^{-1} to the upper mmol L^{-1} range or until solubility was reached at 20°C and pH 6.8. Subsequently, 0.5 mL of each compound dilution solution as well as pure growth substrate for the controls was added to the glass tubes. Each test was performed twice in independent experiments except for N^4-OH-SMX and the mixture experiment as only a limited amount of reference material was available. The starting concentration of the replicate experiments was diluted by 3:4 in growth medium prior to pipetting of the dilution series in order to better describe the dose-response relationship by filling the gaps between the data points of the first experiment. Three negative controls and one positive control were run with each test. According to the ISO method phenol was selected as a positive control for LI and GI, since Zn^{2+} of the alternatively applicable $ZnSO_4$ can precipitate and in this way bias the results.[14] Glass tubes used in the experiments were homogenized using a vortex prior to measurement.

SMX and some of the investigated TPs show slow and low water solubility. Usually, NaOH is added to increase the ionized species and thus, water solubility. However, since significant pH changes can be critical for ecotoxicological testing and comparability, solutions were prepared one day before testing and treated with an ultrasonic heater (max. 30°C). For NO_2- and NO-SMX, dimethylsulfoxide (DMSO) was added as co-solvent (<1%) to the stock solution to enhance solubility. Although DMSO was often reported to be used for

this purpose, its effect on the luminescence and growth of *V. fischeri* was checked before experiments (see below, section "DMSO as co-solvent").

Growth medium interference test. In order to check for possible effects such as toxicity masking between the growth medium and tested target compound, 1:1 dilutions series of the growth medium with distilled water were prepared from 100 % growth substrate as used in the experiments to 6.25 % (five dilutions), while the target compound concentration was kept constant (tested for SMX and SFA). Controls had to be prepared as references for each growth substrate dilution since the latter show different absorbances at 600 nm. SMX and SFA exhibit no absorbance at 600 nm.

S3 Validation test results

Luminescence and growth kinetics. The kinetics of luminescence and growth without any toxicant were tracked on an hourly time-scale (Figure S1, Supporting Information) to determine an adequate test duration. It can be observed that bioluminescence drops to near 0 after 3 hours and increases then strongly reaching a plateau after 20 h, while growth is permanent and the stationary phase has not been reached after 24 h. Consequently, a test duration of 24 h was selected for both endpoints luminescence and growth inhibition.

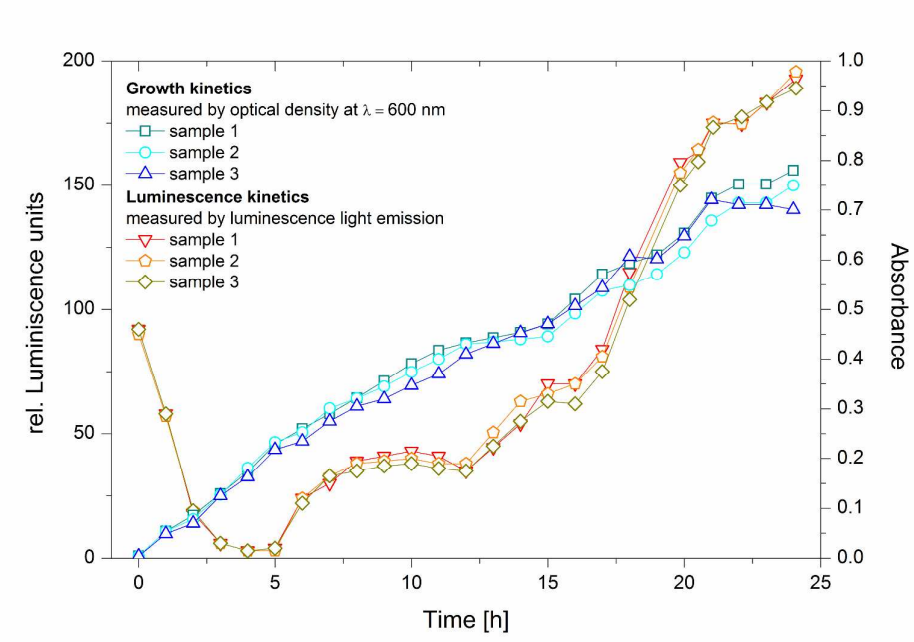


Figure S1. Luminescence (n = 3) and growth (n = 3) kinetics over the applied test duration of 24 h.

Growth medium interference. Since the compounds used in the test validation used by Menz et al. (2013)[14] clearly differ from the molecular features of SMX and its TPs, the influence of the additional growth substrate was exemplarily checked for SMX and SFA. Micropollutants including antibiotics are well known to interact with dissolved and particulate organic matter via non-polar or ionic interactions, which can lead to the masking of their actual toxicological impact. Among the two compounds, SMX is present as around 80% of its ionized species at pH 6.8 (negatively charged sulfonamide group) exhibiting a possible site for ionic interaction while SFA is completely neutral. Apart from that, as sulfonamides act via competitive enzyme inhibition, the ratio between substrate and toxicant may be relevant as well as possible luminescence light absorption by the growth medium itself. Results revealed that there was no significant effect of the substrate concentration from 100 % substrate (no dilution) to 6.3 % (diluted with distilled water) on the luminescence or growth inhibition results (Figure S2, Supporting Information). This was additionally confirmed by the LI_{30min} results of SMX tested using both the ISO medium and the growth medium (Figure S3, Supporting Information). The shape of the dose-response fits differed only marginally and EC_{50} values were in very good agreement with 649 µmol L^{-1} (r^2 = 0.90, n = 18) and 658 µmol L^{-1} (r^2 = 0.99, n = 26), respectively.

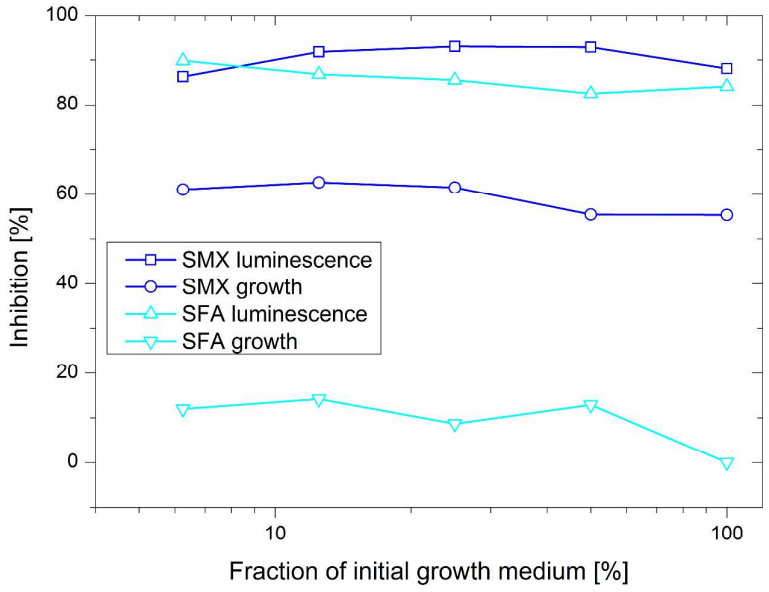


Figure S2. Effect of growth medium concentration on the luminescence and growth inhibition of sulfamethoxazole (SMX) and sulfanilamide (SFA); 100% = initial growth medium concentration used in this study, which was subsequently diluted with distilled water; test duration: 24 h.

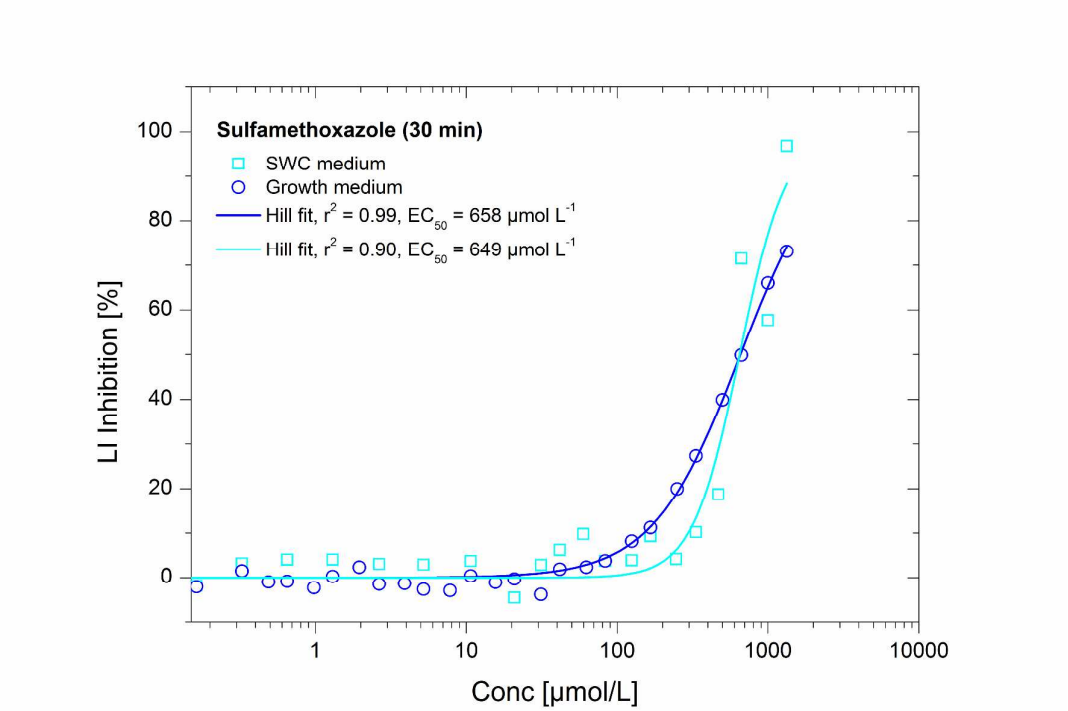


Figure S3. Dose-response relationship of sulfamethoxazole in SWC medium and growth medium using luminescence inhibition of *V. fischeri* over 30 min; each curve represents the results of two independent experiments; concentrations used in the replicate test were selected to fill the data gaps and to obtain a well-defined curve shape; solubility was reached at approximately 1.3 mmol L^{-1}.

DMSO as co-solvent. For both compounds, NO_2-SMX and NO-SMX, DMSO was used as a co-solvent when preparing the solutions. To avoid possible toxic effects, DMSO alone was tested for LI and GI in the growth medium (Figure , Supporting Information). EC_{50} values were found at 5.8 – 5.9 % DMSO solution for LI_{30}, LI_{24} and GI_{24}. Consequently, DMSO concentrations less than 1 % in the final solution were used in samples as well as in the negative controls.

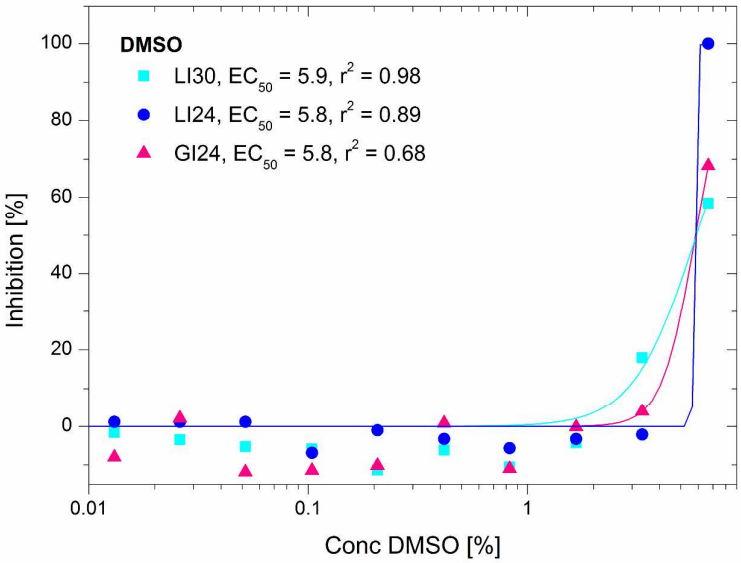


Figure S4. Effect of dimethylsulfoxide (DMSO) on luminescence and growth of *V. fischeri* when used as additional solvent; percentages indicate proportion of DMSO in the solution of the tested compound, which subsequently is added to the *V. fischeri* reagent.

S4 Dose-response relationships of growth inhibition

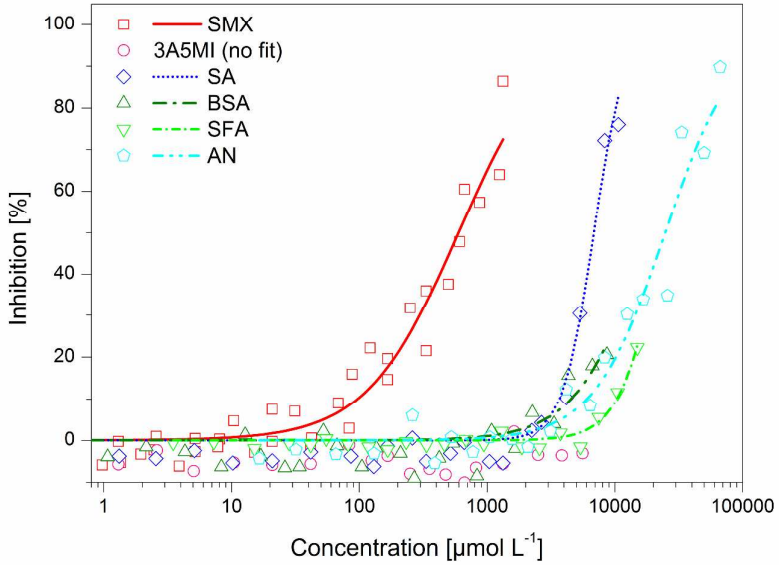


Figure S5. Growth inhibition dose-response relationships of sulfamethoxazole (SMX), 3-amino-5-methyl-isoxazole (3A5MI), sulfanilic acid (SA), benzenesulfonamide (BSA), sulfanilamide (SFA) and aniline (AN).

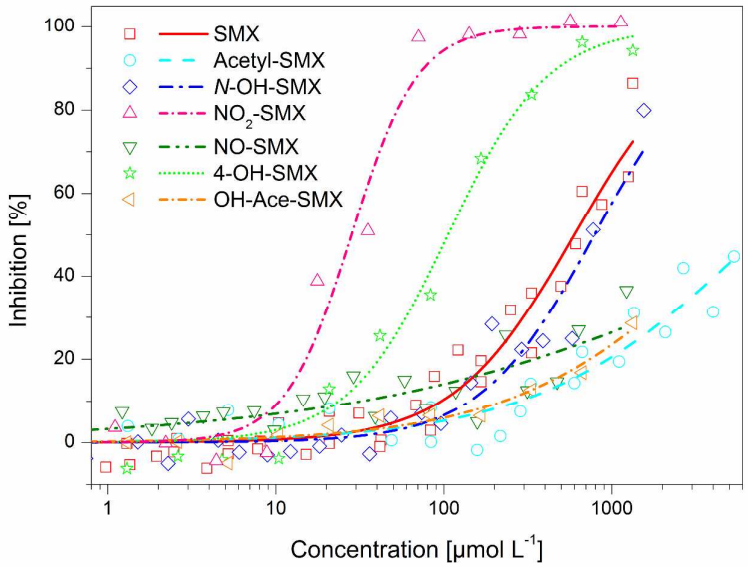


Figure S6. Growth inhibition dose-response relationships of sulfamethoxazole (SMX), N^4-acetyl-sulfamethoxazole (Acetyl-SMX), N^4-hydroxy-sulfamethoxazole (*N*-OH-SMX), 4-nitroso-sulfamethoxazole (NO-SMX), 4-hydroxy-sulfamethoxazole (4-OH-SMX), N^4-hydroxy-acetyl-sulfamethoxazole (OH-acetyl-SMX) and 4-nitro-sulfamethoxazole.

S5 pK_{a2} determination

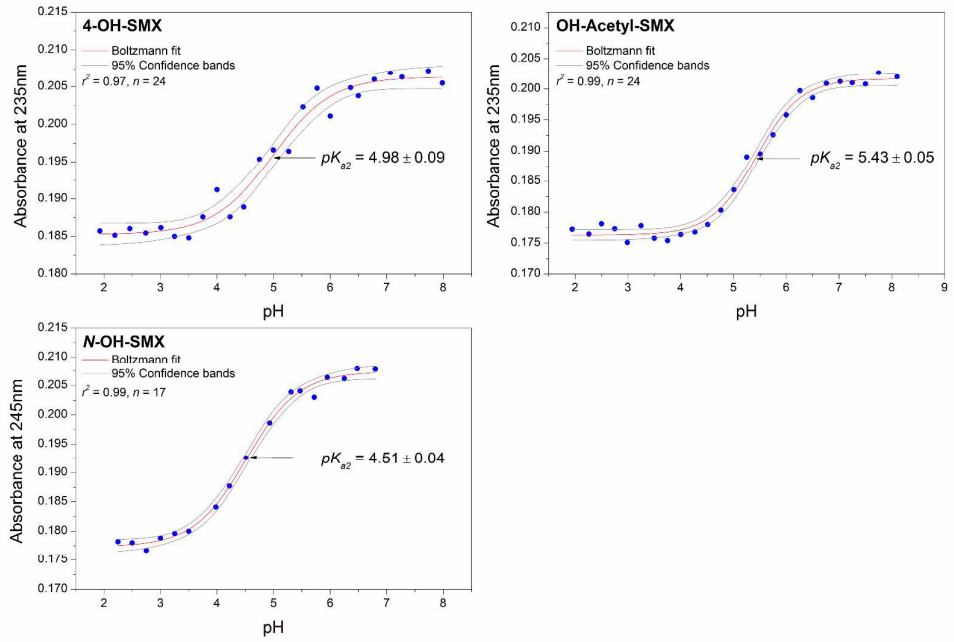


Figure S7. pK_{a2} determination of 4-hydroxy-sulfamethoxazole, N^4-hydroxyl-acetyl-sulfamethoxazole and N^4-hydroxy-sulfamethoxazole; concentrations of the TPs were c = 3 µmol L^{-1} and the temperature was ϑ = 20°C.

References Supporting Information

[1] García-Galán, M. J., Silvia Díaz-Cruz, M., and Barceló, D. (2008) Identification and determination of metabolites and degradation products of sulfonamide antibiotics. *TrAC Trends Anal. Chem. 27*, 1008-1022.

[2] Xu, B., Mao, D., Luo, Y., and Xu, L. (2011) Sulfamethoxazole biodegradation and biotransformation in the water-sediment system of a natural river. *Biores. Technol. 102*, 7069-7076.

[3] Larcher, S., Yargeau, V. (2011) Biodegradation of sulfamethoxazole by individual and mixed bacteria. *Appl. Microbiol. Biotechnol. 91*, 211-218.

[4] Nödler, K., Licha, T., Barbieri, M., and Pérez, S. (2012) Evidence for the microbially mediated abiotic formation of reversible and non-reversible sulfamethoxazole transformation products during denitrification. *Water Res. 46*, 2131-2139.

[5] Barbieri, M., Carrera, J., Ayora, C., Sanchez-Vila, X., Licha, T., Nödler, K., Osorio, V., Perez, S., Kock-Schulmeyer, M., Lopez de Alda, M., and Barcelo, D. (2012) Formation of diclofenac and sulfamethoxazole reversible transformation products in aquifer material under denitrifying conditions: batch experiments. *Sci. Total Environ. 426*, 256-263.

[6] Bonvin, F., Omlin, J., Rutler, R., Schweizer, W. B., Alaimo, P.J., Strathmann, T. J., McNeill, K., and Kohn, T. (2012) Direct photolysis of human metabolites of the antibiotic sulfamethoxazole: evidence for abiotic back-transformation. *Environ. Sci. Technol. 47*, 6746-6755.

[7] Müller, E., Schüssler, W., Horn, H., Lemmer, H. (2013) Aerobic biodegradation of the sulfonamide antibiotic sulfamethoxazole by activated sludge applied as co-substrate and sole carbon and nitrogen source. *Chemosphere 92*, 969-978.

[8] Boreen, A. L., Arnold, W. A. and McNeill, K. (2004) Photochemical fate of sulfa drugs in the aquatic environment: sulfa drugs containing five-membered heterocyclic groups. *Environ. Sci. Technol. 38*, 3933-3940.

[9] Ricken, B., Corvini, P. F., Cichocka, D., Parisi, M., Lenz, M., Wyss, D., Martinez-Lavanchy, P. M., Muller, J. A., Shahgaldian, P., Tulli, L. G., Kohler, H. P., and Kolvenbach, B. A. (2013) Ipso-hydroxylation and subsequent fragmentation: A novel microbial strategy to eliminate sulfonamide antibiotics. *Appl. Microbiol. Biotechnol. 79*, 5550-5558.

[10] Zhou, W., and Moore, D. E. (1994) Photochemical decomposition of sulfamethoxazole. *Inter. J. Pharm.* 110, 55-63.

[11] Trovo, A. G., Nogueira, R. F., Aguera, A., Sirtori, C., and Fernandez-Alba, A. R. (2009) Photodegradation of sulfamethoxazole in various aqueous media: persistence, toxicity and photoproducts assessment. *Chemosphere 77*, 1292-1298.

[12] Richter, D., Dünnbier, U., Massmann, G., Pekdeger, A. (2007) Quantitative determination of three sulfonamides in environmental water samples using liquid chromatography coupled to electrospray tandem mass spectrometry. *J. Chrom. A 1157*, 115-121.

[13] Fröhner, K., Meyer, W., and Grimme, L.H. (2002) Time-dependent toxicity in the long-term inhibition assay with Vibrio fischer. *Chemosphere 46*, 987-997.

[14] Menz, J., Schneider, M., and Kümmerer, K. (2013) Toxicity testing with luminescent bacteria – Characterization of an automated method for the combined assessment of acute and chronic effects. *Chemosphere 93*, 990-996.

[15] PhysProp Database, SRC Inc., http://esc.syrres.com/fatepointer/search.asp, accessed March 6, 2014.

[16] Lin, C.-E., Lin, W.-C., Chen, Y.-C., and Wang, S.-W. (1997) Migration behavior and selectivity of sulfonamides in capillary electrophoresis. *J. Chrom. A 792*, 37-47.