Environ. Sci. Technol. **2004**, *38,* 5607−5615

Transformation of the Antibacterial Agent Sulfamethoxazole in Reactions with Chlorine: Kinetics, Mechanisms, and Pathways

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Sulfamethoxazole (SMX)—a member of the sulfonamide antibacterial class—has been frequently detected in municipal wastewater and surface water bodies in recent years. Kinetics, mechanisms, and products of SMX in reactions with free chlorine ($HOCl/OCl^-$) were studied in detail to evaluate the effect of chlorination processes on the fate of sulfonamides in municipal wastewaters and affected drinking waters. Direct reactions of free available chlorine (FAC) with SMX were quite rapid. A half-life of 23 s was measured under pseudo-first-order conditions ($[FAC]_0 = 20$ μM (1.4 mg/L) and $[SMX]_0 = 2$ μM) at pH 7 and 25 °C in buffered reagent water. In contrast, a half-life of 38 h was determined for reactions with combined chlorine (NH_2Cl, $NHCl_2$) under similar conditions. Free chlorine reaction rates were first-order in both substrate and oxidant, with specific second-order rate constants of 1.1×10^3 and 2.4×10^3 M^{-1} s^{-1} for SMX neutral and anionic species, respectively. Investigations with substructure model compounds and identification of reaction products verified that chlorine directly attacks the SMX aniline-nitrogen, resulting in (i) halogenation of the SMX aniline moiety to yield a ring-chlorinated product at sub-stoichiometric FAC concentrations (i.e., $[FAC]_0$:$[SMX]_0 \leq 1$) or (ii) rupture of the SMX sulfonamide moiety in the presence of stoichiometric excess of FAC to yield 3-amino-5-methylisoxazole, SO_4^{2-} (via SO_2), and *N*-chloro-*p*-benzoquinoneimine. Reaction ii represents an unexpected aromatic amine chlorination mechanism that has not previously been evaluated in great detail. Experiments conducted in wastewater and drinking water matrixes appeared to validate measured reaction kinetics for SMX, indicating that SMX and likely other sulfonamide antibacterials should generally undergo substantial transformation during disinfection of such waters with free chlorine residuals.

Introduction

Sulfamethoxazole (structure shown in Table 1) is an important member of the synthetic sulfonamide antibacterials or sulfa drugs. Sulfonamides have been used extensively for many years in human and veterinary medicines to treat diseases and infections and in feed additives to promote growth rate and weight gain of food animals (*1*), and continue to be prescribed in combination with the antibacterial agent trimethoprim as a major first-line clinical option for treatment of various bacterial infections (*2*). Research in recent years has verified the ubiquity of numerous antibacterial compounds in the aquatic environment (*3, 4*). Among the target analytes measured, various sulfonamide antibacterials—sulfamethoxazole in particular—have been repeatedly detected at concentrations of 70−150 ng/L in surface waters and 200−2000 ng/L in secondary wastewater effluents (*3*−*7*).

The presence of antibacterials in the aquatic environment is of particular concern because of fears that they may stimulate dissemination of antibacterial resistance among native bacterial populations. Though a causal link between chronic exposure to sub-therapeutic concentrations of antibacterials and induction of antibacterial resistance has yet to be proven, singly and multiply antibacterial resistant bacteria have been detected in municipal wastewater effluents, sewage-affected surface water systems, and even drinking water (*8*−*11*). The detection in aquatic environmental systems of resistant bacteria and the antibacterial agents to which they are resistant clearly merits a great deal of concern regarding the fate of antibacterial compounds in relevant water treatment processes.

Aerobic biodegradation of sulfonamide antibacterials is understandably quite limited (*12, 13*), which provides one important indication as to why these compounds might persist within municipal wastewater effluents and surface water bodies. However, reported results of two more recent studies indicate that sulfonamides are quite susceptible to transformation via various chemical oxidation processes involving application of free chlorine or ozone (though no transformation products were identified) (*14, 15*). Observations from a recent occurrence study appear to support the relevance of such transformation at field conditions, as significant losses of sulfonamides were noted during chlorine disinfection processes in several municipal wastewater treatment plants located within the United States (*7, 16*).

Reactions related to sulfonamide chlorination have previously been investigated only to a limited extent (*17*). This prior research was intended to provide an explanation for adverse toxicological reactions associated with human use of such antibacterials and did not address reaction kinetics or investigate reaction mechanisms under conditions likely to be encountered in municipal wastewater or drinking water disinfection. The current investigation was undertaken with the intent of not only quantifying kinetics for reactions of the sulfonamide antibacterial sulfamethoxazole (referred to as SMX subsequently) with free available chlorine (FAC) and combined chlorine (CC) but also identifying associated transformation mechanisms and degradation products relevant to chlorine-based municipal wastewater and drinking water disinfection processes.

As illustrated by SMX (Table 1), sulfonamide compounds contain two moieties connected to both sides of the characteristic sulfonamide linkage ($-NH-S(O_2)-$). Among sulfonamide antibacterials, the aniline moiety in para-connection to the sulfonyl S is present in all, and variations in structures typically stem from the other moiety connected to the sulfonamide N (*1*). Sulfonamides exhibit wo acid dissociation constants; one involving protonation of the aniline N, and the other corresponding to deprotonation of the sulfonamide NH (*18, 19*), as shown in Figure 1. A classical second-order kinetics model (*20*−*22*) incorporating specia-

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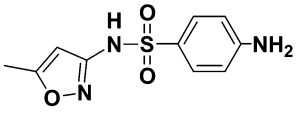
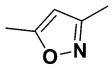
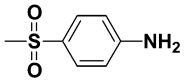
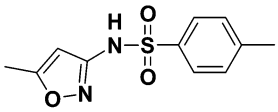
10.1021/es035225z CCC: $27.50
Published on Web 09/30/2004

TABLE 1. Sulfamethoxazole and Associated Substructure Model Compounds

Compound	Structure	Mol. Weight	pK_a
Sulfamethoxazole (SMX)		253.28	pK_{a1} = 1.7, pK_{a2} = 5.6 *(18)*
3,5-dimethylisoxazole (DMI)		97.12	Not Available
4-aminophenyl methyl sulfone (APMS)		171.22	pK_{a1} = 1.5 *(19)*
4-methyl-N-(5-methyl-isoxazol-3-yl)-benzenesulfonamide (MMIB)		252.29	Not Available

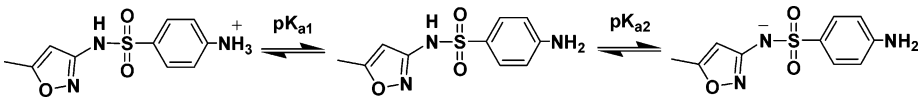


FIGURE 1. Speciation of sulfamethoxazole.

tion of both substrate and free chlorine oxidant was adapted to critically evaluate the effect of pH on SMX/FAC reaction rate. Sub-structural model compounds (Table 1) that correspond to either the hypothesized reactive or non-reactive portions of SMX were used to probe the reactive functional groups. LC/MS, GC/MS, and ^{1}H NMR were utilized in conjunction with supplementary analytical techniques to identify reaction products. All of the results were then combined and analyzed to deduce the reaction mechanisms and pathways related to transformation of SMX by FAC. Additional experiments were conducted in real municipal wastewater and drinking water matrixes to assess field applicability of observations obtained in the laboratory from clean water reaction systems.

Materials and Methods

Standards and Reagents. Sulfamethoxazole (SMX) was obtained from ICN Biomedicals (Irvine, CA). 3,5-Dimethylisoxazole (DMI), 3-amino-5-methylisoxazole (AMI), 4-aminophenyl methyl sulfone (APMS), and 4-aminophenol were purchased from Sigma-Aldrich (St. Louis, MO). 4-Methyl-*N*-(5-methylisoxazol-3-yl)benzenesulfonamide (MMIB) was purchased from ASINEX (Moscow, Russia). All commercially available chemical standards were of at least 98% purity and were used without further purification. *N*-Chloro-*p*-benzoquinoneimine (NCBQ) was synthesized from 4-aminophenol according to the procedure reported by Venuvanalingam et al. (*23*). NaOCl was obtained from Fisher Scientific (Pittsburgh, PA) at ~7% purity. All other reagents used (e.g., buffers, colorimetric agents, reductants, etc.) were obtained from Fisher Scientific (Pittsburgh, PA) and were of reagent grade quality or better. All reagent solutions were prepared using Nanopure purified water (Barnstead, Dubuque, IA).

One hundred milligrams per liter substrate stock solutions (for use in kinetic studies) were prepared in water, with 10% methanol as a cosolvent. One gram per liter SMX stock solutions (for use in LC/MS product characterization studies) were prepared using 50% methanol. Stock solutions were replaced after 2 months of storage at <5 °C. Aqueous sodium hypochlorite stock solution was diluted to yield ~100 mg/L FAC stocks for use in clean water and drinking water kinetic experiments and ~1 g/L FAC stocks for use in wastewater kinetic experiments and product identification studies. FAC stock solutions were standardized iodometrically (*24*). Preformed chloramine stocks were prepared according to Chapin (*25*) at pH 4.5, 5, 6, 7, 8, and 9 in 0.1 M acetate (pH ≤ 5), 0.1 M phosphate (6 ≤ pH ≤ 8), and 0.025 M borate (pH 9) buffers by combining buffered 302 mg/L solutions of NH_4Cl and buffered 200 mg/L solutions of FAC at 25 °C in 1:1 proportion (i.e., at 2:1 [NH_4Cl]:[FAC] molar ratio) under completely mixed conditions. The resulting solutions contained ~100 mg/L of CC. The CC stocks were prepared prior to each experiment, temporarily stored at <5 °C, and used within 24 h of generation. Inorganic chloramine concentrations were standardized using DPD-FAS titrimetry (*24*).

TABLE 2. Wastewater and Drinking Water Sample Characteristics

water quality data	pH	alkalinity (mg/L, as $CaCO_3$)	NH_3 (mg NH_3-N/L)	DOC (mg C/L)
wastewater	7.3	120	<0.12[a]	14.0
drinking water	6.6	13	<0.12[a]	1.3

[a] Minimum detectable concentration was ~120 μg NH_3-N/L (~7 × 10^{-6} mol/L).

Municipal Wastewater and Drinking Water Samples. Reactions of SMX with FAC were studied in wastewater and drinking water process samples to gauge potential effects of matrix on the reactions. The procurement and sources of these water samples are described in the Supporting Information (Text S1). Important characteristics of these water samples are listed in Table 2. These samples represent low-NH_3 process waters that are typically subjected to disinfection by chlorine oxidants.

Reaction Monitoring by Analysis of Substrate Loss. An Agilent 1100 series HPLC system equipped with a Zorbax RX-C18 column (4.6 mm × 250 mm, 5 μm), thermostat (30 °C), and UV diode-array detector was used to monitor loss of parent compounds and product formation during kinetic measurements. Detection wavelengths for SMX, DMI, APMS, and MMIB were set at 275, 210, 210, and 205 nm, respectively.

Depending on the chromatographic behavior of respective analytes, gradient and isocratic methods were used with varying ratios of 0.04 M phosphate buffer (containing 1:1 $[H_3PO_4]$:$[NaH_2PO_4]$) to pure acetonitrile. Sample injection volumes of 100 μL were used in all cases, and typical quantification limits for analytes under these conditions were ~100 nM. Standard curves yielded coefficients of determination (r^2) greater than 0.99 within the range of experimental concentrations in all cases. Recoveries of SMX after spiking into real water samples were 100 ± 5%. Experiments performed to monitor reactions of SMX in clean water systems were conducted in triplicate, while all other experiments were conducted in duplicate. Oxidant controls were included in all experimental sets to ensure that substrates were stable in the absence of added FAC or CC.

Clean Water Experiments. Reaction solutions contained within 30-mL, amber, borosilicate glass bottles were partially immersed in a 25 °C recirculated water tank and stirred with Teflon-coated stir bars using a 15-position magnetic stir-plate. Experiments were conducted at pH values between 4 and 9. Ten millimolar acetate (pH 4, 4.5, and 5), phosphate (pH 6, 6.5, 7, and 8), and borate (pH 9) buffers were used to maintain pH in all reagent water experiments. Final pH of reaction solutions did not vary by more than 0.1 units from starting values.

Reactions were initiated by adding appropriate volumes of FAC or CC stock (to achieve at least a 10:1 $[oxidant]_0$:$[substrate]_0$ ratio) to solutions containing 500 μg/L of substrate. One Milliliter samples were subsequently taken at evenly-spaced time intervals and analyzed by HPLC-UV to follow loss of substrate. Loss of SMX in the presence of CC was monitored by HPLC−UV immediately after sampling, without quenching. Reaction times for CC and SMX were corrected by taking into account the dead-volume of the HPLC system to calculate the exact time at which substrates were separated from the inorganic chloramine oxidants. In all other cases, 1-mL samples of each reaction solution were quenched at appropriate time intervals to remove excess FAC. Sodium thiosulfate was used to quench residual FAC in experiments with DMI, while samples of MMIB were analyzed both with and without thiosulfate quenching. A "soft" quenching technique using NH_4Cl was utilized to consume residual FAC in experiments with SMX and APMS. Additional details of the latter quenching procedure are included in the Supporting Information (Text S2). FAC and CC residual concentrations were measured by DPD colorimetry or DPD-FAS titrimetry (*24*) at the conclusion of each kinetic experiment. Oxidant concentrations did not generally decrease by more than 10% from starting concentrations during the course of these experiments. Even in cases of greater chlorine loss, however, pseudo-first-order plots of substrate loss remained satisfactorily linear (see below).

Municipal Wastewater and Drinking Water Experiments. Real water matrix experiments were conducted without modification of solution pH. Twenty milliliter volumes of each real water sample were spiked with 500 μg/L of SMX and dosed with FAC concentrations selected to approximate those utilized by the plant from which they were derived, which yielded free chlorine residuals of 1−2 mg/L. Reactions were followed in the same manner as for the clean systems described above, except that oxidant decay was also taken into account by measuring residual FAC concentrations at selected intervals during and after corresponding monitoring periods.

Calculation of Pseudo-First-Order Rate Constants and Verification of Reaction Orders. Plots of ln([substrate]) versus time were linear in all kinetic experiments, with r^2 values ranging from 0.95 to 1.0. Pseudo-first-order rate constants k'_{app} were obtained from the slopes of regression lines of such plots. The 95% confidence limits are reported with k'_{app} for SMX as error bars in relevant graphs. Additionally, the relationship between measured k'_{app} and dose of FAC was found to be linear within the range of 10:1 to 25:1 $[FAC]_0$:$[SMX]_0$ ratios. A log−log plot of $[FAC]_0$ against k'_{app} for SMX yielded a line with a slope close to 1, indicating that this reaction can be treated as first-order with respect to FAC. The SMX/FAC reaction can therefore be described as a bimolecular, second-order reaction: substrate + FAC → products.

Product Identification. *LC/MS Analysis of Reaction Product Mixtures.* SMX was added to 0.1 M pH 7 phosphate buffer to achieve starting concentrations of 100 mg/L. FAC solution was subsequently added to initiate reactions at oxidant:substrate molar ratios ranging from 1:2 to 4:1. Unquenched sample aliquots were immediately analyzed by an Agilent 1100 series LC/MS system equipped with a Zorbax SB-C18 column (2.1 mm × 150 mm, 5 μm), thermostat (30 °C), UV diode-array detector, and a single-quadrupole mass spectrometer. Analyte peaks were resolved using gradient elutions with varying ratios of 0.2% (v/v) formic acid to pure acetonitrile. MS analyses were conducted using positive mode electrospray ionization (ESI^+), over a mass scan range of 50−1000 m/z. The mass spectrometer fragmentation voltage was typically set to 80 eV, unless additional fragmentation of molecular ions was desired, in which case this voltage was set to 120 eV. Spray chamber temperature and drying gas flow were set to 325 °C and 10 L/min, respectively.

Fraction Collection and LC/MS Confirmation of Products Observed during Kinetic Studies. Because the HPLC columns and analytical method parameters used for SMX kinetic studies (HPLC/UV/FLD) and product identification (LC/MS) were different, identities of product peaks observed during kinetic monitoring were investigated by collecting corresponding HPLC fractions and analyzing these by the LC/MS methods described above (additional details in the Supporting Information, Text S3).

^{1}H NMR and GC/MS Analyses of Product SMX2. ^{1}H NMR and GC/MS were utilized to provide structural information for one degradation product of SMX not identifiable by LC/MS (additional details in the Supporting Information, Text S4).

Additional Analyses. Sulfate was qualitatively monitored by precipitation with $BaCl_2$ and subsequent measurement of turbidity (*24*).

Results and Discussion

Kinetics of Reactions with Free Available Chlorine. Apparent pseudo-first-order rate constants (k'_{app}) observed for reactions of SMX with FAC were clearly dependent on solution pH (Figure 2). This behavior was evaluated by modeling relative contributions of individual oxidant and antibacterial substrate species to the overall second-order reaction described by

$$\frac{d[substrate]_{tot}}{dt} = -k'_{app}[substrate]_{tot} = -k''_{app}[oxidant]_{tot}[substrate]_{tot} \quad (1)$$

where k''_{app} represents the apparent second-order rate constant for the overall reaction.

Solution pH affects the speciation of both free chlorine oxidant and antibacterial substrate. HOCl can be modeled as a typical monoprotic acid, using eq 2 and standard equilibrium distribution equations for α_1(HOCl) and $\alpha_2(OCl^-)$:

$$[oxidant]_{tot} = [HOCl] + [OCl^-] = \sum_{i=1,2} \alpha_i [oxidant]_{tot} \quad (2)$$

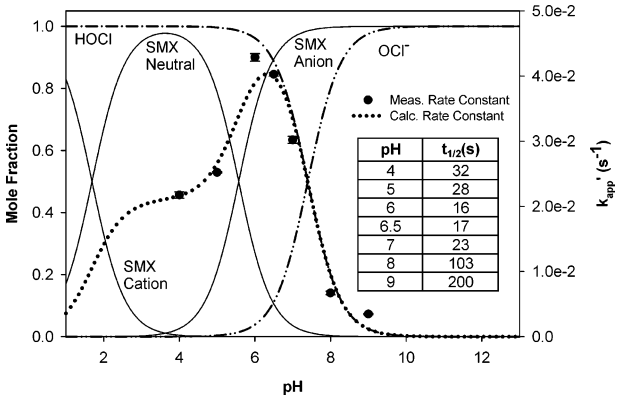


FIGURE 2. pH dependency of apparent pseudo-first-order rate constants for reactions of SMX with FAC ($[SMX]_0 = 2.0 \times 10^{-6}$ M, $[FAC]_0 = 2.0 \times 10^{-5}$ M (1.4 mg/L), $T = 25$ °C).

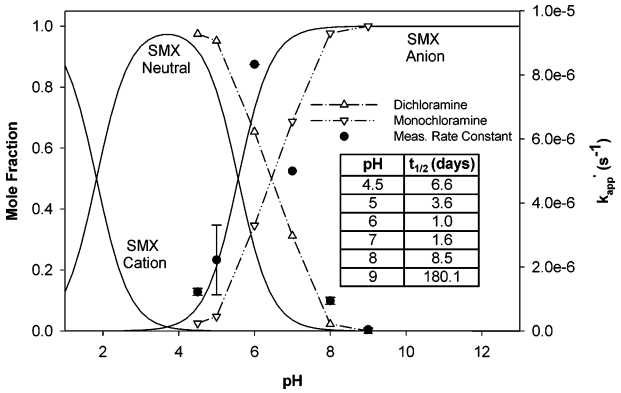


FIGURE 3. pH dependency of apparent pseudo-first-order rate constants for reactions of SMX with CC ($[SMX]_0 = 2.0 \times 10^{-6}$ M, $[CC]_0 = 2.0 \times 10^{-5}$ M (1.4 mg/L), $T = 25$ °C).

SMX can be modeled as a typical diprotic acid by applying eq 3 and standard equilibrium distribution equations for α_1' (cationic SMX), α_2' (neutral SMX), and α_3' (anionic SMX):

$$[\text{substrate}]_{tot} = [H_2A^+] + [HA] + [A^-] = \sum_{j=1,2,3} \alpha_j'[\text{substrate}]_{tot} \quad (3)$$

Incorporation of these oxidant and substrate speciation relationships into eq 1 yields the general expressions shown in eqs 4 and 5:

$$\frac{d[\text{substrate}]_{tot}}{dt} = -\sum_{\substack{i=1,2 \\ j=1,2,3}} k_{ij}\alpha_i[\text{oxidant}]_{tot}\alpha_j'[\text{substrate}]_{tot} \quad (4)$$

$$k_{app}'' = -\sum_{\substack{i=1,2 \\ j=1,2,3}} k_{ij}\alpha_i\alpha_j' \quad (5)$$

where k_{ij} represents the specific second-order rate constant for reaction of oxidant species i with substrate species j. Apparent second-order rate constants (k_{app}'') can be determined using eq 1, where k_{app}' (in s^{-1}) $= k_{app}''[\text{oxidant}]_{tot}$ and k_{app}'' (in M^{-1} s^{-1}) $= k_{app}'/[\text{oxidant}]_{tot}$.

The general reaction kinetics model represented by eqs 4 and 5 can be simplified by critically evaluating apparent contributions of each oxidant or substrate species to the rate constants presented in Figure 2. The reactivity of SMX with FAC dropped off sharply with decreasing abundance of HOCl, suggesting that SMX rate constants are dominated by reactions involving HOCl and that OCl^- does not contribute significantly to the overall reactivity of SMX with FAC. Similar observations—that HOCl (rather than OCl^-) represents the primary oxidant species in FAC reactions—can be found in the literature (*20*−*22*, *26*). Reactions with OCl^- have thus been neglected in model calculations. In addition, reaction of HOCl with the cationic form of SMX has been neglected on the basis of two assumptions: first, abundance of the SMX cation is rather low within the pH ranges studied (pK_a for the aromatic amine is 1.7, which is 2.3 pH units lower than the lowest pH value studied); second, protonation of the aniline's primary amino group should at the very least retard (if not prevent) the reaction between HOCl and SMX by coordinating the lone-pair electrons associated with this nitrogen. The kinetics for reaction of SMX with HOCl can therefore be modeled by

$$\frac{d[\text{SMX}]_{tot}}{dt} = (-k_{12}\alpha_1\alpha_2' - k_{13}\alpha_{13}')[\text{oxidant}]_{tot}[\text{substrate}]_{tot} \quad (6)$$

The specific rate constants k_{ij} for reactions among HOCl and each individual substrate species were calculated by least-squares regression of the experimental data. The regression was performed by using the Solver function in Microsoft Excel to minimize the square of the difference between measured and predicted values via optimization of the k_{ij} values. One constraint was imposed on these calculations to permit determination of specific second-order rate constants with this approach: that is, substrate species exhibiting a higher degree of functional group deprotonation were assumed to be equally or more reactive toward HOCl than their more fully protonated structural variants (in agreement with findings from prior investigations of pH-dependent chlorination reaction rates; *20*−*22*, *26*). The k_{12} (i.e., reaction of HOCl with neutral SMX) and k_{13} (i.e., reaction of HOCl with anionic SMX) values obtained with this procedure are 1.1×10^3 and 2.4×10^3 M^{-1} s^{-1}, respectively.

As a check on the assumptions made in developing eq 6, a regression of the experimental data was also performed with respect to the general model (eq 4). This regression yielded values close to 0 for the constants k_{ij} corresponding to reactions omitted from the simplified models. Exclusion of these values did not significantly affect model accuracy. Application of eq 6—using the calculated k_{12} and k_{13} values—allows one to successfully model the observed pH dependencies of apparent pseudo-first-order rate constants k_{app}' for SMX as shown in Figure 2. This provides additional support for the assumptions made in model development.

Kinetics of Reactions with Combined Chlorine. Reactions of SMX with CC were much slower than with FAC (Figure 3). Minimum measured half-life was 1 d for reactions of SMX (pH 6) under pseudo-first-order conditions ($[CC]_0$:$[\text{substrate}]_0$ = 10). The 180-d half-life determined for SMX at pH 9 indicates that reactions with monochloramine are extremely slow and that CC reaction rates observed at lower pH were dominated by dichloramine. These observations agree with expected oxidation strengths for each oxidant species, where $HOCl/OCl^-$ > $NHCl_2$ > NH_2Cl (*27*). This indicates that reactions of SMX with CC are unlikely to be important in municipal wastewater or drinking water disinfection processes—where monochloramine typically represents the primary chloramine disinfectant—unless breakpoint chlorination is practiced, in which case reaction rates should still be quite slow.

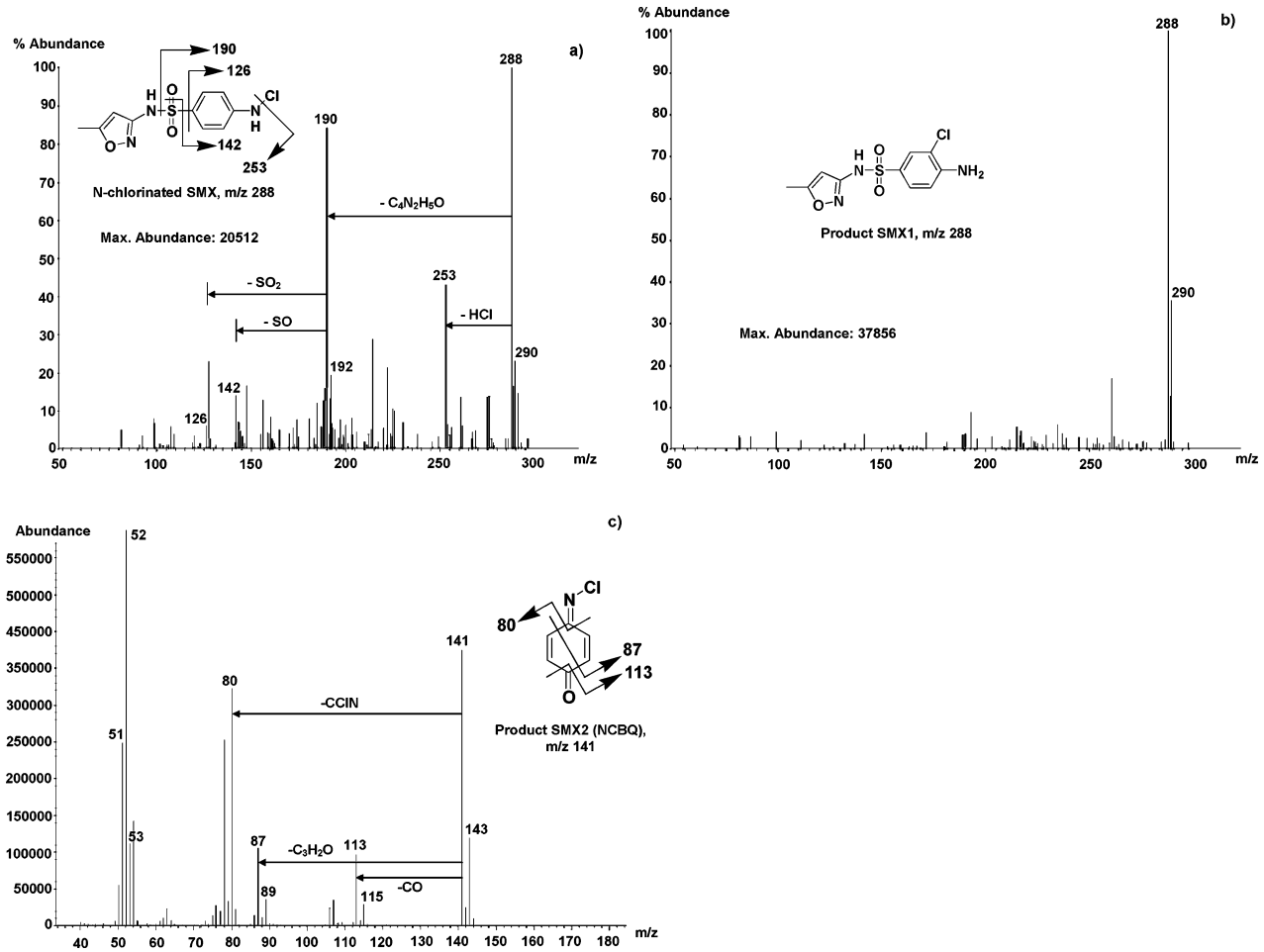


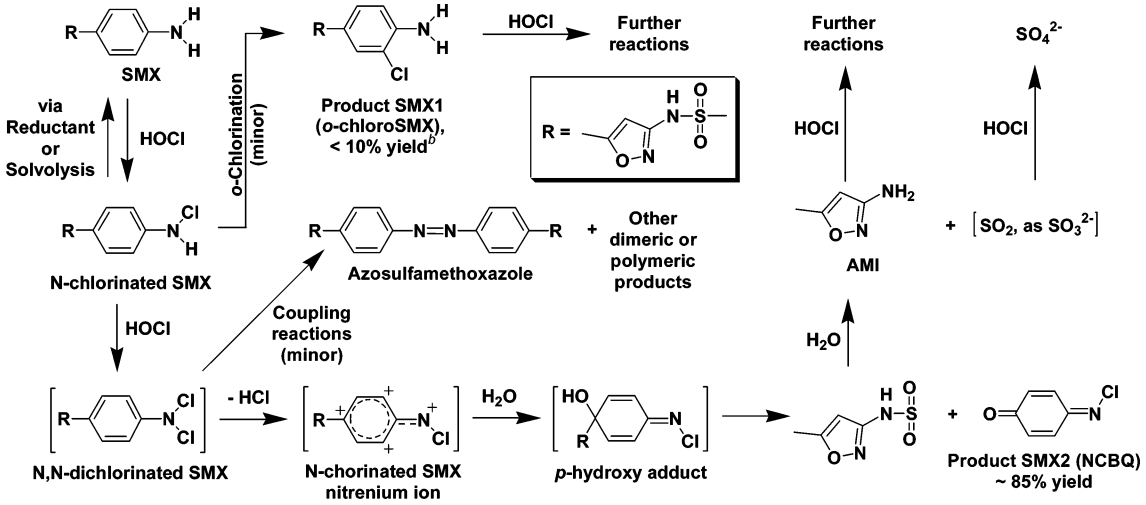
FIGURE 4. Mass spectra for major products of the reaction between SMX and FAC: (a) N-chlorinated SMX [LC/MS spectrum obtained at 120 eV fragmentation voltage], (b) Product SMX1, and (c) Product SMX2. Spectra a and b were obtained by LC/MS-ESI$^+$, and spectrum c was obtained by GC/MS-EI. Characteristic LC/MS fragment ions were identified according to Hartig et al. (*5*); GC/MS fragments were postulated according to McLafferty and Tureček (*54*).

Reactivity of Substructure Compounds and Identification of Reaction Centers. Reactions of substructure compounds with FAC were examined at pH 4, 7, and 9 using pseudo-first-order conditions ($[FAC]_0$:$[substrate]_0 = 10$). DMI and MMIB were nonreactive with FAC (no measurable decrease in concentration over 30 to 90 min), while APMS was quite reactive ($k'_{app} = 2.5 \times 10^{-2}$, 2.2×10^{-3}, and 9.8×10^{-4} s^{-1} at pH 4, 7, and 9, respectively, in the presence of 20 μM FAC). The reactivity of APMS (which contains the aniline amino-nitrogen but no sulfonyl amido-nitrogen) and apparent non-reactivity of MMIB (which contains the sulfonyl amido-nitrogen but no aniline amino-nitrogen) toward FAC indicate that direct attack of the SMX molecule by FAC occurs at its aniline moiety rather than at its amido-nitrogen. Furthermore, the non-reactivity of DMI indicates that the 5-methylisoxazole moiety of SMX does not play an important role in reactions with free chlorine. The observed pH dependency of SMX reaction kinetics (where anionic SMX appeared to be more reactive toward FAC than neutral SMX (Figure 2)) suggests that, although direct reaction occurs at SMX's aniline moiety, this reaction is modulated by protonation equilibria of the SMX amido-nitrogen. Protonation of the amido-nitrogen in the neutral SMX species should enhance the electron-withdrawing strength of SMX's *p*-sulfonamide group relative to the corresponding anionic species, which would in turn reduce the nucleophilicity of the molecule's aromatic system by inductive and resonance effects, thereby reducing its susceptibility to attack by FAC.

Product Identification. Transformation products described here pertain only to reactions involving SMX and FAC. Reactions with CC appeared to be unimportant with regard to water treatment, as discussed earlier, and so were not investigated in further detail. Two products observed in LC/MS-ESI$^+$ analyses of under-chlorinated SMX/FAC reaction mixtures ($[FAC]_0$:$[SMX]_0 < 1$)—both exhibiting molecular ions of m/z 288 (with isotopic peaks at 290)—appeared to represent N-chlorinated and ring-chlorinated SMX molecules (Figure 4a,b). The former product appeared to be reduced to SMX upon treatment with $Na_2S_2O_3$ (Supporting Information Figure S1), hence its description as an N-chlorinated structure. The latter compound (Product SMX1) was detected only at very low yield, so its behavior could not be investigated in a similar manner. However, comparison with results from the prior study of SMX chlorination (*17*) and consideration of expected aniline halogenation patterns (*28*) support the structural assignments.

A number of additional products were observed in solutions dosed with stoichiometric excess of FAC at $[FAC]_0$:$[SMX]_0 > 1$ (Supporting Information Table S1). A prominent, early-eluting peak detected by LC/MS was identified as 3-amino-5-methylisoxazole (AMI) (refer to Scheme 1 for structure) by comparison to a standard of this compound. Small amounts (as indicated by small MS signal areas) of apparent dimeric or polymeric products were also detected by LC/MS, although only one appeared to be identifiable, as azosulfamethoxazole (Scheme 1 and Supporting Information

SCHEME 1. Proposed Pathways for Reactions of SMX with FAC[a]



[a] Structures in brackets are given as probable intermediates. [b] Estimated yield obtained from data reported by Uetrecht et al. (*17*).

Table S1). In addition to AMI and dimeric/polymeric products, Product SMX2 was observed by UV detection ($\lambda_{max} \approx 290$ nm) but yielded no signal in positive APCI or ESI modes of LC/MS analyses. Product SMX2 was isolated via solid-phase extraction methods (Supporting Information Text S4) and analyzed by GC/MS and ^{1}H NMR.

GC/MS analyses of Product SMX2 (Figure 4c and Supporting Information Table S1) showed a molecular ion of m/z 141 (chlorine isotope peak at m/z 143) and suggested that the structure could be *N*-chloro-*p*-benzoquinoneimine (NCBQ), which is an intermediate in the Berthelot reaction for analysis of ammonia (*29*) and belongs to a family of compounds commonly applied in the Gibbs reaction for colorimetric analysis of phenolic compounds in aqueous solution (*30*, *31*). The UV spectrum and observed half-life (~48 h) for decay of a Product SMX2 isolate in aqueous solution matched those previously reported for NCBQ (*29*). In addition, the apparent reduction of Product SMX2 by sodium thiosulfate and its ability to oxidize potassium iodide were consistent with such a structure.The ^{1}H NMR spectrum of Product SMX2 (Supporting Information Figure S3) was also compatible with the NCBQ structure, where (i) signals corresponding to the methyl group and heterocyclic proton of SMX's isoxazole ring (as observed for pure SMX) were absent from the spectrum of Product SMX2 and (ii) instead of two aromatic proton signals (as observed for pure SMX), the spectrum of Product SMX2 exhibited four proton signals, presumably due to anisotropic effects created by the presence of an *N*-chlorimino group (*32*). Final confirmation of Product SMX2 as NCBQ was obtained from comparison of the former compound's UV spectrum and HPLC retention time to those observed for a standard of the latter. Quantification of NCBQ in HPLC monitoring showed the ultimate yield of this transformation product to be approximately 85% when FAC was present in greater than 2-fold molar excess of SMX.

Qualitative measurement of SO_4^{2-} in solutions dosed with excess FAC and varying concentrations of SMX indicated that SO_2 is also evolved during the reaction of FAC with SMX (note that all SO_2 would undergo hydration to yield sulfite, which would be quantitatively oxidized to SO_4^{2-} in the presence of excess FAC; *33*). Taken together with the products presented in the preceding paragraphs, the results indicate that SMX's sulfonamide structure is disrupted in reactions of SMX with excess FAC, yielding NCBQ, AMI, and SO_2. None of these products were mentioned in the prior study of SMX chlorination (*17*). However, review of that study shows that reactions were only evaluated in cases for which SMX was present in substantial molar excess of FAC.

Proposed Reaction Pathways. Observations pertaining to apparent ortho-chlorination of the SMX aniline ring in solutions dosed with $[FAC]_0$:$[SMX]_0 < 1$ are in agreement with results reported previously by Uetrecht et al. (*17*). These investigators suggested that N-chlorinated SMX rearranged to ortho-chlorinated SMX during analytical time frames of roughly 1−2 h. Ring-chlorination of anilines has previously been demonstrated to proceed primarily through intermediate N-chlorinated aniline structures (*28*, *34*). In the current study, repeated analyses of unquenched under-chlorinated reaction solutions showed that a decrease in the N-chlorinated SMX peak observed in HPLC chromatograms corresponded to an increase in the SMX peak. This suggests that N-chlorinated SMX also undergoes a back-reaction in the absence of reducing agents—to yield the parent SMX—over a time scale of several hours. This is in accord with results previously obtained by Gassman and Campbell (*35*), which indicated that N-chlorinated anilines containing electron-withdrawing para-substituents undergo solvolytic decay to yield relatively high proportions (10−30%) of the parent compounds, in addition to ring-chlorinated products.

The current findings related to generation of NCBQ, AMI, and SO_2 in over-chlorinated SMX solutions point toward an additional reaction mechanism. Evaluation of SMX/FAC product evolution patterns showed that loss of SMX was accompanied by an initial increase in the concentration of N-chlorinated SMX (Figure 5). As mentioned in the preceding paragraph, N-chlorinated SMX decayed slowly over a span of 1 to 2 h in the *absence* of excess FAC (*17*). However, product evolution observations obtained in the current investigation indicated that N-chlorinated SMX decayed within seconds in the *presence* of excess FAC (Figure 5). In the latter case, decay of N-chlorinated SMX was followed by an increase in abundance of NCBQ. Quite notably, however, the decrease in abundance of N-chlorinated SMX did not appear to correlate directly with the evolution of NCBQ. NCBQ signal area continued to increase even after all N-chlorinated SMX had reacted. These observations indicate that the appearance of NCBQ is likely preceded by formation and decay of an additional intermediate.

Scully and co-workers (*36*−*38*) reported quite extensively that N,N-dichlorinated amino acids—which were generated from amino acids in the presence of molar excesses of FAC—decayed primarily to *N*-chlorimine products. Similarly, synthesis of NCBQ can be achieved by dosing solutions of 4-aminophenol with excess FAC (*23*, *30*). The above reactions can likely be extrapolated to other aromatic amine compounds such as aniline or SMX. Thus, it seems reasonable

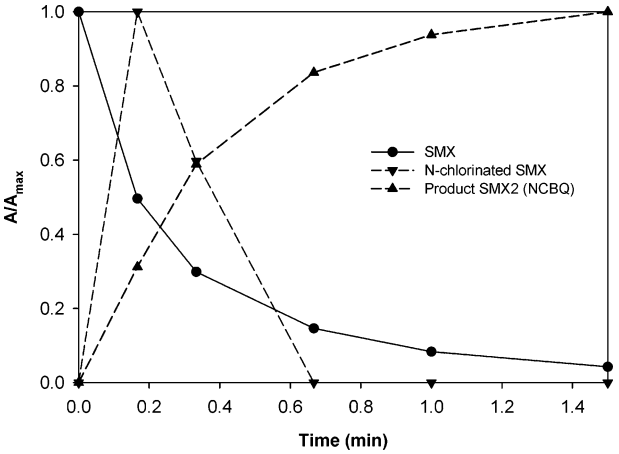


FIGURE 5. Product evolution for reactions of SMX with FAC ($[SMX]_0 = 2.0 \times 10^{-6}$ M, $[FAC]_0 = 2.0 \times 10^{-5}$ M (1.4 mg/L), pH 6.5, $T = 25$ °C). Samples were quenched according to the NH_4Cl method described in the Supporting Information. A/A_{max} represents the ratio of peak area at any given time to maximum measured peak area at 275 nm.

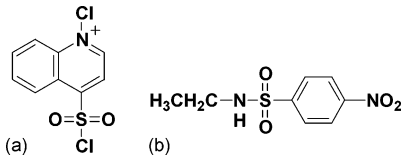


FIGURE 6. Structures previously found to be susceptible to sulfonyl group displacement by nucleophilic attack: (a) 4-chloroquinoline sulfonyl chloride (*39*) and (b) PNU-15189 (*40*).

to propose that further chlorination of N-chlorinated SMX leads to formation of a N,N-dichlorinated SMX intermediate, which subsequently decays to yield the *N*-chlorimine NCBQ as well as AMI and SO_2 (Scheme 1). Failure to detect the N,N-dichlorinated SMX intermediate may have been a result of typical 2−4 min lag times between sampling and instrumental analysis, which could have allowed sufficient time for its decay prior to detection. The mechanism by which decay of N,N-dichlorinated SMX could lead to cleavage of the sulfonamide moiety requires additional explanation, as discussed below.

Kwart and Body (*39*) observed facile cleavage of the S−C bond during chlorination of 4-quinoline sulfonyl chloride (structure provided in Figure 6a), which they attributed to production of electron-deficient character in the heterocyclic quinoline's nitrogen by attack of chlorine. They suggested that delocalization of this electrophilic character within the substrate's aromatic system led to expulsion of the sulfonyl group via facilitation of nucleophilic attack at the α-carbon adjacent to the sulfur atom (para to the heterocyclic nitrogen). A more recent study showed that cleavage of sulfonamides could be readily achieved by enzyme-mediated nucleophilic attack at the α-carbon of the benzenesulfonamide moiety in compounds possessing very strong electron-withdrawing substituents para to the sulfonamide group (e.g., the structure shown in Figure 6b; *40*). In each of these studies, the presence of a strongly electrophilic carbon center α- to the sulfonyl or sulfonamide groups' S was cited as essential for the S−C bond cleavage to occur.

An arylnitrenium cation (Scheme 1, see ref *41* for an overview)—which can be generated by heterolysis of aromatic chloramines' N−Cl bonds (*42, 43*)—is compatible with the requirement stated above and could provide an explanation for the observations noted here with respect to SMX/FAC reactions. Substantial positive character is delocalized from an arylnitrenium's cationic nitrogen atom into its aromatic system, resulting in distribution of strong electron-deficiency to the aromatic ring's para and ortho positions. Arylnitrenium species are known to exhibit extremely high rate constants ($\sim 10^8$−10^{10} M^{-1} s^{-1}) for reactions with nucleophiles such as N_3^-, Cl^-, Br^-, and H_2O, where nucleophilic attack occurs preferentially at the carbon atom para to the divalent amino nitrogen (*44, 45*). Aqueous reactions of para-substituted nitrenium ions typically lead to a *p*-hydroxy intermediate, which can subsequently rearrange to *p*-benzoquinoneimine structures (*46, 47*). As proposed in Scheme 1, H_2O may attack at the para position of a N-chlorinated SMX nitrenium species (produced by heterolytic decay of N,N-dichlorinated SMX), resulting in displacement of the sulfonamide group by water. This reaction would be expected to yield NCBQ and an unstable AMI−SO_2 complex. The AMI−SO_2 complex would decay readily in the presence of water to yield AMI and SO_2 (*40*). Both AMI and SO_2 (after hydration to SO_3^{2-}) would then be expected to react subsequently with FAC. Although products of AMI chlorination were not explicitly investigated, SO_3^{2-} would clearly be oxidized to SO_4^{2-} (*33*).

An alternate explanation for the generation of NCBQ, AMI, and SO_2 might involve direct nucleophilic attack by H_2O or OH^- at the para position of N,N-dichlorinated SMX via a more conventional S_N2 substitution. In this case, nucleophilic addition would produce heterolysis of a N−Cl bond via intramolecular redistribution of electrons to yield the chlorimine rather than vice versa, as described in the preceding paragraph. Unfortunately, the similar expected product distributions of this and the nitrenium mechanism make explicit confirmation of either a difficult task. However, the results obtained by Scully and co-workers (*36*−*38*) provide a clue as to which process actually governs the decay of SMX. Their studies showed that decay of N,N-dichlorinated primary amino acids was initiated by heterolysis of one N−Cl bond, which led to generation of the *N*-chlorimine product. No products were detected that would have suggested that hydrolytic attack occurred at electrophilic α-carbons adjacent to the dichloramine groups *prior* to N−Cl heterolysis. The sequence of reaction steps (i.e., N−Cl heterolysis followed by nucleophilic substitution and chlorimine formation) depicted in Scheme 1 is in analogy to the findings in these previous studies (*36*−*38*).

Azosulfamethoxazole (Scheme 1) presumably forms via a coupling reaction involving N,N-dichlorinated SMX (since N-chlorinated SMX was only observed to react back to SMX or to rearrange to Product SMX1; *17*), similar to polymerization reactions previously reported to occur during chlorination of aromatic amines (*48*−*50*). Whether this reaction occurs via a radical mechanism (*48, 51*) or through reactions involving singlet nitrene species (*47*) is unclear. However, the high yields of NCBQ (~85%) indicate that the dimerization reaction is not likely very important in the overall SMX/FAC reaction scheme. Furthermore, generation of coupling products is probably even less significant in systems containing lower SMX concentrations typical of environmental samples.

In light of these results, one could deduce that SMX/FAC reactions should contribute at least to partial reduction or elimination of the parent compound's antibacterial activity (derived from its antagonistic competition with *p*-aminobenzoic acid for dihydropteroate synthase enzyme, which is necessary for bacterial folic acid synthesis; *52*). Mechanisms leading to disruption of the sulfonamide structure (shown in Scheme 1) could very likely lead to a loss of this activity from the parent compound, although the resulting products, particularly NCBQ, might possess higher acute toxicity to aquatic organisms than the parent substrates. On the other hand, the effects of aromatic ring-chlorination—in which the *p*-aminobenzenesulfonamide structure is essentially conserved—are less clear.

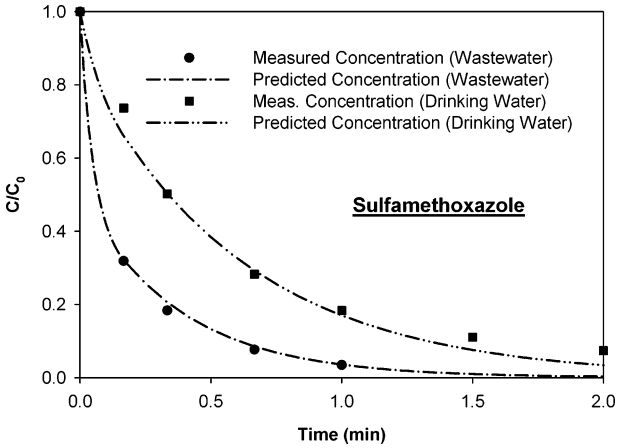


FIGURE 7. Reaction kinetics of SMX in real waters (T = 25 °C). Wastewater: pH 7.3, $[SMX]_0 = 2.0 \times 10^{-6}$ M, $[FAC]_0 = 1.6 \times 10^{-4}$ M (11 mg/L); drinking water: pH 6.6, $[SMX]_0 = 2.0 \times 10^{-6}$ M, $[FAC]_0 = 2.8 \times 10^{-5}$ M (2 mg/L).

The findings presented here are particularly important in two respects. First, the chlorination reaction mechanisms elucidated for SMX are most likely applicable to other sulfonamide antibacterials because the reactive *p*-aminobenzenesulfonamide moiety is common to all members of this structural family. Second, results of the current study are quite significant in understanding general reactivities of aromatic amine-containing compounds toward free chlorine. Direct electrophilic ring-chlorination has been previously suggested as the predominant transformation pathway in aqueous reactions of aromatic amine structures with FAC (*53*). However, in the most comprehensive prior investigation to address aqueous chlorination of anilines, the authors used a maximum $[FAC]_0$:$[substrate]_0$ ratio of 1.5 (*51*). As a consequence, formation of *N*,*N*-dichloroanilines would have been minimal, so that associated products would have been generated at relatively low yields and may have escaped detection. The results of one additional investigation indicated that ring-chlorinated products accounted for only 30% of total aniline loss (*50*). Furthermore, the authors of the latter study suggested production of polymeric substances such as indoaniline, which forms through a reaction pathway involving NCBQ (*49*)—during extended reaction periods. Taken into context with the current results, a re-evaluation of aqueous aniline−chlorine chemistry at higher $[FAC]_0$:$[substrate]_0$ ratios more typical of water treatment seems warranted.

Studies in Real Water Matrixes. Reactions of SMX in real water samples were modeled by assuming two different reaction steps: one characterized by rapid decay of FAC during the first 10 s (due to rapid consumption by highly reactive substrates such as reduced sulfur compounds, amines, etc. in the water samples), and one by slower FAC decay during the remaining reaction time. FAC decay during the two reaction periods was treated as pseudo-first-order and was modeled using FAC residual measurements taken at various time intervals during the experimental monitoring periods.

Observed oxidation kinetics for SMX corresponded quite closely to those predicted by the second-order model after consideration of concurrent FAC decay (Figure 7). Product evolution patterns in environmental matrix experiments (observed via HPLC/UV) were also qualitatively similar to those observed in clean systems.

The rates at which SMX decayed in these real water samples indicate that substantial conversion of the parent compound can be expected for residence times typical of wastewater (5−30 min) and drinking water clearwells (1−24 h), since the reaction appears to be initiated by attack of a single HOCl molecule on a single substrate molecule. This expectation is supported by existing field-scale observations (*7*, *16*).

However, two cautionary notes should be added to this discussion. First, reaction pathways for SMX proceed through a reducible intermediate, which can be converted to the parent compound upon treatment with strong nucleophiles such as SO_3^{2-} or $S_2O_3^{2-}$. This suggests that in North American wastewater treatment facilities, at which dechlorination with reduced sulfur compounds is commonly practiced after chlorine disinfection, the parent compound could be regenerated from the N-chlorinated intermediate prior to final discharge of wastewater effluent into the environment. This could provide one explanation for the detection of SMX in North American surface waters (*4*), for which the main points of entry are likely those associated with municipal wastewater discharges. Second, as discussed above, N-chlorinated SMX was observed to decay either by ring-chlorination (<10%) or by back-reaction to the parent SMX. This suggests that, in cases where insufficient free chlorine is added to treated waters to initiate the sulfonamide rupture mechanism described herein (Scheme 1), a significant fraction of the N-chlorinated intermediate may simply react back to SMX. Such behavior could provide an additional explanation for the frequent detection of SMX within affected surface water bodies.

Finally, emphasis must be placed on the fact that the real water matrixes used in this study were typical of low-NH_3 process waters. In each case, the FAC dose applied was at least 2-fold greater (on a molar basis) than measurable NH_3 concentrations, so that a FAC residual was present. Underchlorination of process waters containing excess NH_3 would not likely lead to significant loss of SMX, because NH_3 should out-compete SMX for FAC (on the basis of expected reaction rates for NH_3 with FAC at near-neutral pH; *22*), and SMX does not react appreciably with CC.

Additional investigations will be necessary to more fully assess implications of the current work, particularly with respect to the significance of sulfonamide degradation products. In general, future monitoring studies addressing the environmental fate of antibacterials should also begin to take into account the potential presence of degradates resulting from the chemical transformation mechanisms presented here, since it is possible that perceived elimination of parent antibacterials in treated waters might not always correspond to elimination of their biologically active structural moieties.

Acknowledgments

This study was funded by the Georgia Water Resources Institute. Financial support for M.C.D. from the U.S. Environmental Protection Agency STAR fellowship and the Georgia Tech Foundation is gratefully acknowledged. The authors thank Jaehong Kim, James Mulholland, Les Gelbaum, and Gretchen Onstad for providing helpful suggestions in discussions of this work as well as Urs von Gunten, Marc Huber, and Amisha Shah for their helpful comments and continuous support. In addition, the time and insightful recommendations of three reviewers are highly appreciated.

Supporting Information Available

Text, figures, and tables addressing (1) real water sample procurement, source characteristics, and parameter measurements; (2) reaction quenching procedures; (3) supplemental means of product identification; and (4) principal reaction products identified by LC/MS and ^{1}H NMR. This material is available free of charge via the Internet at http://pubs.acs.org.

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Received for review November 4, 2003. Revised manuscript received July 27, 2004. Accepted August 16, 2004.

ES035225Z