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Chemical and toxicological evolution of the antibiotic sulfamethoxazole under ozone treatment in water solution

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ABSTRACT

This work studied the elimination paths of the sulfonamide antibiotic sulfamethoxazole by ozonation in fast kinetic regime. The ozonation runs were performed in conditions favouring either the direct attack of the ozone molecule or the indirect attack by ozone-generated radical species with initial concentration of 0.150 mM. When doses of ozone were transferred to the liquid phase 0.2 mM, in no case did sulfamethoxazole remain in solution. Two main transformation pathways were found involving the preferential attack of molecular ozone or radical pathway and leading to the formation of six intermediates, which were identified by LC-ESI-QTOF-MS. Both routes took place simultaneously in the different conditions tested, leading to a hydroxylation reaction of the benzene ring, oxidation of the amino group on the benzene ring, oxidation of the methyl group and the double bond in the isoxazole ring and S–N bond cleavage. The most abundant reaction intermediate was that resulting from S–N bond cleavage. The toxicity of partially ozonated samples for *Daphnia magna* and *Pseudokirchneriella subcapitata* revealed the formation of toxic by-products during the early stages of reaction and the persistence of considerable toxicity after the total depletion of sulfamethoxazole.

1. Introduction

The incomplete elimination of pharmaceuticals and other emerging pollutants in conventional wastewater treatment plants has provoked their widespread distribution in the environment [1]. Some of the adverse effects of these substances for ecosystems and non-target species have been reported but in many cases still remain unknown [2,3]. There have been great efforts in recent years to develop, apply and evaluate the effectiveness of additional or alternative treatments for wastewater. Advanced oxidation processes (AOP) are a broad group of aqueous phase oxidation methods based on the generation of highly reactive species, such as hydroxyl radicals, which allow the depletion of organic pollutants in dilute solution [4]. Ozone based methods, with ozone as the only oxidant or in association with other oxidants may involve the selective attack of molecular ozone to certain organic moieties or the non-selective reaction with ozone-generated free hydroxyl radicals. The production of hydroxyl radicals can be enhanced by raising pH or by combining ozone with hydrogen peroxide or UV-irradiation [5]. The efficiency of ozone-based AOP is closely related to ozone dose, wastewater composition and the nature of the target organic compounds [6,7]. Although ozonation leads to the elimination of many organic compounds in aqueous solution, this is not necessarily accompanied by total mineralization [4]. In most cases, degradation by-products generated in the process persist after the parent compounds have been totally eliminated, a fact which highlights the need to characterize reaction mixtures in order to identify persistent and possibly toxic compounds. Recently, it has been shown that ozonation may release oxidation intermediates with enhanced toxicity for aquatic life [8,9]. Stalter et al. [10] found a significant inhibition of the reproduction of the annelid *Lumbriculus variegatus* in contact with ozonated wastewater that indicated the formation of toxic oxidation byproducts. They also reported that sand filtration after ozonation reduces toxic effects to the level of conventional treatments, an interesting result with practical repercussion associated with biological degradation in biologically active filter systems. Dantas et al. monitored the biodegradability and toxicity of a solution of sulfamethoxazole during an ozonation treatment in conditions of moderate mineralization [11]. Their results showed an increase in biodegradability accompanied by a rise in acute toxicity for *Vibrio fischeri* during the first thirty minutes of ozonation.

The identification of unknown transformation products is not an easy task and very often requires the combined use of several

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analytical techniques and strategies. The use of LC–MS, combined with a new generation of MS systems, has great advantages for the analysis of polar compounds. They allow sensitive analysis and provide abundant structural information for elucidating unknown structures. Triple quadrupole (QqQ) or linear ion trap (QqLIT or QTRAP) analyzers involve transformation product elucidation on the basis of structural information gained in tandem MS/MS experiments, whereas the measurement of accurate mass and subsequent determination of the empirical formula provided by time-of-flight (TOF) or quadrupole time-of-flight (QqTOF) instruments area very valuable information source when assigning structures. All these techniques have been widely applied to the identification of metabolites and transformation products generated by different water treatments.

In this work, the ozonation of the antibiotic sulfamethoxazole (SMX) was studied under different operational conditions. SMX is neither degradable nor adsorbable on sewage sludge. Moreover, it was identified in wastewater from a wastewater treatment plant with concentration as high as 370 ng/L and showed a poor removal during biological treatment that consisted in A2O multistage with nitrification–denitrification and enhanced phosphorus removal [12]. Its low K_d value explains the occurrence and transport of SMX in natural waters and previous studies have evidenced its resistance to natural attenuation in the subsurface environment, a fact which augurs the long-term exposure of the microbial community to antimicrobial compounds [13]. As noted earlier, the relatively high cost of reagents and energy in AOP forces a balance to be struck between the target degree of mineralization and the quality required for the effluent. This work evaluates the application of LC-QqTOF-MS to the identification of SMX transformation products generated under three ozonation conditions, at acidic pH, at basic pH and at a combination of basic pH and hydrogen peroxide. The toxicity of partially oxidized mixtures for *Daphnia magna* and *Pseudokirchneriella subcapitata* was assessed using standardized bioassays. The results were related to the identified intermediates and to the amount of ozone transferred to the liquid.

2. Experimental

2.1. Materials

Sulfamethoxazole (99%), p-chlorobenzoic acid (pCBA, 99%), hydrogen peroxide (30%, w/v) and catalase (from bovine liver) were supplied by Sigma–Aldrich (Steinheim, Germany). Solutions were prepared with high purity water obtained from a Milipore Mili-Q system with a resistivity of at least 18 $\mu\Omega\,cm^{-1}$ at 25 °C. Acetonitrile and water HPLC grade were supplied from Merck (Darmstadt, Germany). Formic acid (purity, 98%) was obtained from Fluka (Buchs, Germany).

2.2. Chemical analyses

The concentration of dissolved ozone was monitored using a membrane-covered amperometric Rosemount 499AOZ analyzer that consists of a gas-permeable membrane that covers a gold cathode. Ozone diffuses through the membrane and reacts inside the sensor with the electrolyte solution to form an intermediate compound that is reduced over the cathode producing a current, which the analyzer measures via a Rosemount 1055 SoluComp II Dual Input. Up to our knowledge there is no interference with SMX or with other organic compounds dissolved in the liquid. The analyzer was calibrated against the standard Indigo Colorimetric Method (SM 4500-O3B). Signals corresponding to the concentration of dissolved ozone, pH and temperature were recorded using an Agilent 34970 Data Acquisition Unit connected to a computer. Based on the dynamic response of the three measuring devices, the sampling period was set at 5 s. The concentration of ozone in the gas phase was measured using a non-dispersive UV Photometer Anseros Ozomat GM6000 Pro. The analyses of SMX were performed by HPLC Agilent 1200 with automatic injector G1329A and diode-array UV-VIS detector G1316A, using a Phenomenex C18 150 mm column. The mobile phase was a mixture of acetonitrile and water (40:60) adjusted to pH 3 using ortho-phosphoric acid with an isocratic flow of 1.0 mL/min at room temperature. Detection was accomplished at 270 nm. Total organic carbon (TOC) was determined by means of a Shimadzu TOC-VCSH analyzer equipped with an ASI-V autosampler.

2.3. Analysis by LC-QqTOF-MS

A liquid chromatography-electrospray ionisation-quadrupole-time-of-flight mass spectrometry (LC-ESI-QTOF-MS) system, in positive mode, was used to identify transformation products (TP). The HPLC system was equipped with a reversed-phase XDB-C_{18} analytical column of 4.6 mm × 50 mm, 1.8 μm particle size (Agilent Technologies). 0.1% formic acid and 5% MiliQ water in acetonitrile was used as mobile phase A and 0.1% formic acid in water (pH 3.5) as mobile phase B. The chromatographic method held the initial mobile phase composition (10% A) constant for 1 min, followed by a linear gradient to 100% A in 11 min, and kept for 6 min at 100% A. Flow rate 0.6 mL/min, injection volume 20 μL.

The HPLC system was connected to an Accurate Mass Q-TOF MS (Agilent 6530 Series) operating in the 4 GHz High Resolution Mode. Ions were generated using an electrospray ion source with Agilent Jet Stream Technology. Operation conditions were: superheated nitrogen sheath gas temperature (400 °C), capillary, 4000 V; nebulizer, 40 psi; drying gas, 5 L/min; gas temperature, 350 °C; skimmer voltage, 65 V; fragmentor 90 V.

The mass axis was calibrated over the m/z 40–3200 range. MS/MS spectra were acquired over the m/z 40–950 range at a scan rate of 0.5 s per spectrum. Data recorded were processed with Agilent MassHunter Workstation Software (version B.02.00).

2.4. Toxicity tests

The immobilization tests for *D. magna* (Daphtoxkit F™ magna, Creasel, Belgium) were conducted following the standard protocol described in the European Guideline [14]. The dormant eggs were incubated at 20 ± 1 °C under continuous illumination of 6000 lx. Between hatching and test steps, the daphnids were fed with the microalgae *Spirulina* to avoid mortality. The pH of samples was adjusted to be in the tolerance interval of the test organisms, that is 7.0 ± 1.0 for *D. magna* and 8.0 ± 1.0 for *P. subcapitata* [15,16]. Test plates with neonates were incubated for 24–48 h in the dark at 20 °C. Acute toxicity was assessed by observing the mobility of *D. magna*. The neonates were considered immobilized if they did not resume swimming within a period of 15 s. Acute toxicity is expressed as percent immobilization compared to a non-toxic control. The chronic toxicity was determined in line with the algal growth inhibition test as per OECD TG 201 *P. subcapitata* open system using 96-well microplates in which the algae were cultured in a total volume of 200 μL. The samples were diluted in culture media until reaching a growth inhibition of about 10% for non-ozonated samples. The medium was prepared using the required amount of concentrated OECD medium to ensure the same concentration of salts in all samples and controls. The growth of *P. subcapitata* was monitored daily for 72 h and assessed by chlorophyll fluorescence (Excitation 444 nm – Emission 680 nm) using a Fluoroskan Ascent FL plate luminometer. Algae beads and culture media were purchased from Microbiotest Inc. Microplates were maintained at 22 °C inside a growing chamber with controlled light intensity

(~100 μmol photons m^{-2} s^{-1}) and humidity levels, with no culture media evaporation and with periodical shaking and aeration. For both organisms and for each sample, toxicity determination was replicated four times in four independent series of assays.

2.5. Ozonation experiments

The ozonation runs were carried out at pH 2 and 8 in a 5-L glass jacketed reactor operating in semi-batch mode. Additional runs at pH 8 were performed while pumping a solution of hydrogen peroxide of 0.013 M continuously injected into the reactor with a flow of 3.0 mL min^{-1} during 10 min. The initial concentration of SMX in ozonated samples was 0.150 mM. This hydrogen peroxide flow was chosen to keep the overall H_2O_2/O_3 molar ratio below 0.5, which corresponds to the overall stoichiometry of the O_3/H_2O_2 reaction, thereby avoiding an excess of hydrogen peroxide and the subsequent hydroxyl radical scavengers [17–19]. pCBA was used as probe compound to assess the presence of hydroxyl radicals in the reaction mixture. The temperature was kept at 25 °C using a Huber Polystat cc2 and monitored throughout the runs by means of a Pt100 Resistance Temperature Detector (RTD). Ozone was produced by a corona discharge ozonator (Ozomatic, 119 SWO100) fed by an AirSep AS-12 PSA oxygen generation unit at a rate of 60 L/h of gas flow containing 8.5 g/Nm^3 ozone, was bubbled into the reactor through a porous glass disk. The gas flow rate was chosen to ensure an almost constant concentration of ozone in gas phase. The reaction vessel was agitated with a Teflon four-blade impeller at 1000 rpm. The mass transfer coefficient was determined in transient runs with pure water. Throughout the runs, certain samples were withdrawn for analysis at prescribed intervals. Additional details on the experimental set-up and procedure are described elsewhere [20]. Residual hydrogen peroxide was removed from pH $8/H_2O_2$ samples before analysis by the addition of 5 μL of 0.5% catalase aqueous solution into 10 mL samples as described elsewhere [21].

3. Results

In the absence of any control, pH decreased during runs by about one pH unit due to the formation of acidic intermediates. For kinetic analysis, pH was controlled by adding NaOH and in this case it was kept inside ±0.2 units throughout the run. The kinetics of a heterogeneous gas–liquid process is controlled by the relative rates of absorption and the chemical reaction. A characterization of kinetic regimes is given by the Hatta number, which represents the maximum rate of chemical reaction relative to the maximum rate of mass transfer [22]. For a second order reaction the Hatta number is expressed as:

$$Ha = \frac{\sqrt{z k_{O_3} \; c_{SMX} \; D_{O_3}}}{k_L} \quad (1)$$

where D_{O_3} is the diffusivity of ozone in water (1.77×10^{-9} m^2 s^{-1}), k_{O_3} the homogeneous second order rate constant for the depletion of the organic compound, z the stoichiometric coefficient, and c_{SMX} the concentration of SMX in the bulk at a given time. The value of the mass transfer coefficient, $k_L = 5.5 \times 10^{-5}$ m s^{-1}, was evaluated according to Calderbank and Moo-Young [23]. The second order rate constant for the direct ozonation reaction, in the order of 10^5 M^{-1} s^{-1}, has been reported elsewhere [11,24,25]. For ozone doses below 0.2 mM, $4 < Ha < 8$, representing a fast kinetic regime where ozone is consumed near the gas–liquid interphase. Accordingly, the concentration of ozone concentrations in solution was typically below 1% of the equilibrium concentration, $c^*_{O_3}$. Fig. 1 shows the evolution of SMX at pH 2 and 8, the later with and without the addition of hydrogen peroxide as a function of the amount

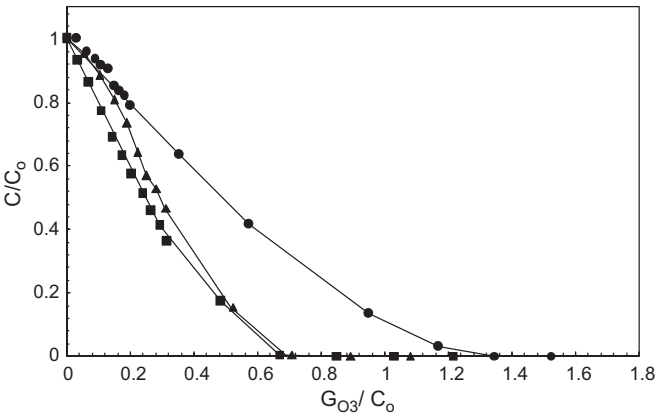


Fig. 1. Variation of dimensionless SMX concentration (C/C_o) as a function of the amount of ozone transferred to the liquid per mole initial SMX, G_{O_3}/C_o for runs pH 2 (●), pH 8 (■) and pH $8/H_2O_2$ (▲).

of ozone transferred to the liquid. The moles of ozone transferred, G_{O_3}, were determined using the following expression:

$$G_{O_3}(t) = E k_L a \int_0^t (c^*_{O_3} - c_{O_3}) dt \quad (2)$$

where k_La is the volumetric mass transfer coefficient and E the enhancement factor calculated as shown elsewhere [26]. In particular, for $Ha > 3$:

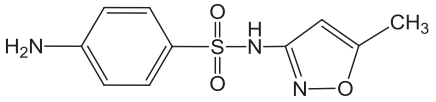
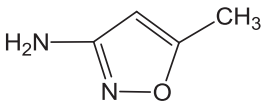
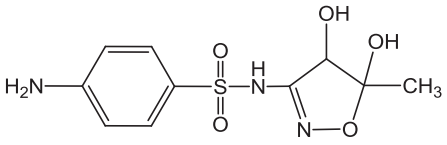
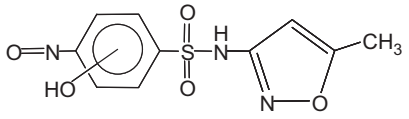
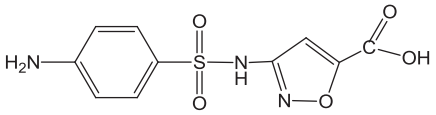
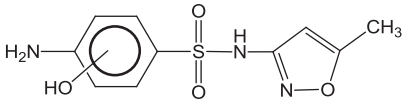
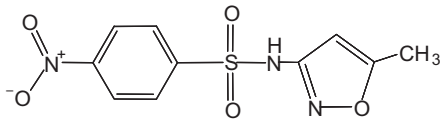
$$E = \frac{Ha}{th(Ha)} \quad (3)$$

The stoichiometric coefficient, z, for the direct ozonation reaction was taken from Dantas et al. [11]. The higher rate observed for SMX removal at pH 8 is most probably due to the enhanced generation of hydroxyl radicals. The relative significance of molecular and mixed molecular/hydroxyl radical attack was assessed by pCBA depletion. In runs performed at pH 2 we did not appreciate any change in pCBA concentration, while for pH 8 and pH $8/H_2O_2$, pCBA decreased by 60–70% after 10 min, revealing the presence of hydroxyl radicals. The differences in pCBA depletion with the use of hydrogen peroxide were not significant, a result in agreement with the fact that SMX removal at pH 8 was not enhanced by the use of hydrogen peroxide. This suggests that in the absence of dissolved ozone in the bulk, the removal of SMX takes place as a result of hydroxyl radicals diffusing from the interface. At the end of the ozonation time (10 min), the removal of TOC was not significantly different from zero at pH 2 and raised to 5% at pH 8. This fact is coincident with the data from Dantas et al. [11] and supposes that even when the ozonation presents a high efficiency to remove SMX, it is not able to mineralize its oxidation byproducts. The use of hydrogen peroxide at pH 8 increased TOC removal to 23% after 10 min on stream, still limited in comparison with the mineralization efficiency observed for this system when treating wastewater [15].

3.1. Identification and kinetic of transformation products (TPs)

Ozonation is not necessarily accompanied by the total mineralization of target organics. In most cases, the degradation by-products generated during the process persist after the total elimination of the parent compounds. This fact highlights the need to characterize the reaction mixtures in order to identify persistent and possibly toxic compounds formed therein. LC-QqTOF-MS analyses were performed to identify TPs generated under the operational conditions proposed (pH 2, pH 8 and pH $8/H_2O_2$). For their identification, information about MS/MS fragmentation pattern

Table 1
Accurate mass measurements of SMX and its TP as determined by LC-ESI (+)-QqTOF-MS in MS/MS mode and structures proposed for the TP identified.

Comp.	Ion mass	Ion formula	Δ Mass (ppm)	DBE	Structure
SMX	**254.0592**	**$C_{10}H_{12}N_3O_3S$**	**0.85**	**7**	
	188.0821	$C_{10}H_{10}N_3O$	−1.24	8	
	160.0866	$C_9H_{10}N_3$	1.85	7	
	156.0113	$C_6H_6NO_2S$	0.44	5	
	108.0447	C_6H_6NO	−2.99	5	
	99.0555	$C_4H_7N_2O$	−2.27	3	
	92.0499	C_6H_6N	−4.93	5	
TP-99	**99.0554**	**$C_4H_7N_2O$**	**−1.27**	**3**	
	72.0448	C_3H_6NO	−5.74	2	
	71.0615	$C_3H_7N_2$	−8.28	2	
	55.0183	C_3H_3O	−7.55	3	
TP-288	**288.0654**	**$C_{10}H_{14}N_3O_5S$**	**−0.06**	**6**	
	270.0559	$C_{10}H_{12}N_3O_4S$	−3.97	7	
	206.0931	$C_{10}H_{12}N_3O_2$	−3.15	7	
	188.0821	$C_{10}H_{10}N_3O$	−1.47	8	
	160.0864	$C_9H_{10}N_3$	3.39	7	
	156.0110	$C_6H_6NO_2S$	2.14	5	
	108.0450	C_6H_6NO	−0.74	5	
TP-284a	**284.0334**	**$C_{10}H_{10}N_3O_5S$**	**0.43**	**8**	
	220.0708	$C_{10}H_{10}N_3O_3$	4.05	8	
	174.0651	$C_9H_8N_3O$	5.99	8	
	160.0497	$C_8H_6N_3O$	5.46	8	
	99.055	$C_4H_7N_2O$	2.8	3	
TP-284b	**284.0339**	**$C_{10}H_{10}N_3O_5S$**	**0.57**	**8**	
	202.0614	$C_{10}H_8N_3O_2$	1.14	9	
	174.0674	$C_9H_8N_3O$	−3.94	8	
	146.0724	$C_8H_8N_3$	−4.39	7	
	105.0442	$C_6H_5N_2$	9.47	6	
TP-270	**270.0541**	**$C_{10}H_{12}N_3O_4S$**	**0.91**	**7**	
	204.0764	$C_{10}H_{10}N_3O_2$	1.51	8	
	172.0057	$C_6H_6NO_3S$	3.34	5	
	124.0394	$C_6H_6NO_2$	−0.85	5	
	108.0445	C_6H_6NO	−1.04	5	
	99.0555	$C_4H_7N_2O$	−2.11	3	
TP-284c	**284.0340**	**$C_{10}H_{10}N_3O_5S$**	**0.27**	**8**	
	220.0708	$C_{10}H_{10}N_3O_3$	3.76	8	
	193.0621	$C_9H_9N_2O_3$	−7.06	7	
	189.0292	$C_9H_5N_2O_3$	4.22	9	
	173.0720	$C_{10}H_9N_2O$	−3.12	8	
	143.0613	$C_9H_7N_2$	−3.16	8	
	122.0233	$C_6H_4NO_2$	6.81	6	

and the calculated formulae of both protonated molecules $[M+H]^+$ and ion fragments were analyzed. This information is set out in Table 1, together with data regarding relative mass error and DBE (double bond and ring equivalents).

The accurate mass spectrum of SMX is shown in Fig. 2. The protonated molecular ion $[M+H]^+$ at m/z 254 was selected as precursor ion to yield the characteristic MS^2 fragmentation pattern showed. The structures proposed for the fragment ions are also depicted in Fig. 2. It is observed how in most cases they arise from rearrangements in the aminobenzene moiety and loss of the SO_2 group and isoxazole ring, the latter resulting in the ion at m/z 99.0555 ($C_4H_7N_2O$). The observation of the characteristic fragmentation of the parent drug usually provides valuable information for the identification of TPs. Thus, the appearance of common fragments in the TP spectra may indicate the prevalence of a specific part of the original molecule, suggesting that the transformation takes place in another part of the structure. This is the case for compound TP-270, which elemental composition, an oxygen atom more than SMX with no alteration of DBE value, indicated the formation of a hydroxylated derivative. The proposed position of the hydroxyl group is the result of observing the fragmentation pattern (Table 1), which showed ion fragments for the aniline ring moiety similar to those obtained for the SMX but containing an additional oxygen atom, such as $C_{10}H_{10}N_3O_2$ (m/z 204.0764) instead of $C_{10}H_{10}N_3O$ (m/z 188.0821), $C_6H_6NO_3S$ (m/z 172.0057) instead of $C_6H_6NO_2S$ (m/z 156.0113) or $C_6H_6NO_2$ (m/z 124.0394) instead of C_6H_6NO (m/z 108.0447). On the contrary, the ion at m/z 99.0555, which corresponded to the isoxazole ring ($C_4H_7N_2O$), remained unchanged, thus confirming the fact that the addition of the hydroxyl group takes place in the benzene ring.

Similar reasoning was applied to the identification of TP-288 (m/z 288.0654). This compound presented a fragmentation pattern similar to SMX, with common fragments at m/z 156.0110 and 108.0450, but with the absence of the ion at m/z 99.0555. This suggested that the SMX underwent a transformation that affected the isoxazole ring. The empirical formula ($C_{10}H_{14}N_3O_5S$) was consistent with the formation of a dihydroxylated compound resulting from the oxidation of the double bond on the isoxazole ring, as confirmed by the one unit reduction observed in the DBE (see Table 1). The consecutive losses of two water molecules (−18 uma) to yield fragments at m/z 270 ($C_{10}H_{12}N_3O_4S$) and m/z 160 ($C_{10}H_{10}N_3O$) also confirm the formation of a diol. This compound was also reported in photo-Fenton [27] and TiO_2 [28] photocatalysis of SMX and its formation can be explained by the formation of a ternary carbon-centered radical, as proposed by Hu et al. [28], and the further electrophilic addition of a second •OH.

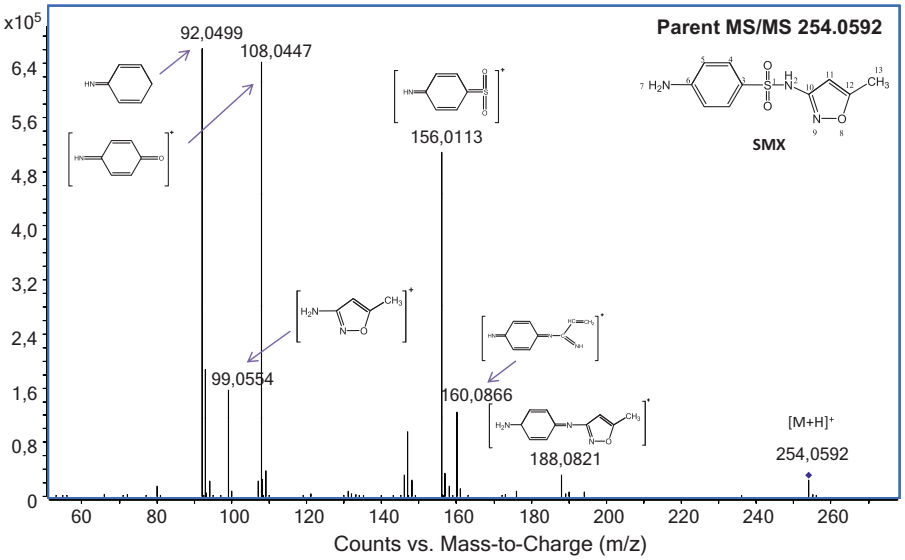


Fig. 2. Product ion spectrum of SMX obtained by QqTOF-MS/MS and proposed fragmentation pathway.

Another interesting finding was the identification of three isobaric compounds. They presented the same accurate mass (m/z 284.0341) and molecular composition ($C_{10}H_{10}N_3O_5S$) but the difference in their elution behaviour indicated a different structure. This was confirmed by comparing the fragmentation pattern of the MS^2 spectra, different for the three compounds, as shown in Fig. 3. No common fragments with the parent drug spectrum were observed except for TP-284a, which exhibited the ion at m/z 99.0550, a characteristic of the isoxazole moiety.

Considering that ozone is a high selectivity electrophile, which mainly reacts with double bonds, activated aromatic systems and non-protonated amines, oxidation of the p-sulfoaniline moiety was expected to take place. This is in agreement with previous studies that report the formation of nitrobenzene as the majority by-product formed after the ozonation of aniline at acid pH [29]. Abellán et al. [30] also report the formation of nitrosulfamethoxazole as the primary degradation product of SMX ozonation. This structure was assigned to the compound TP-284c since this was the one most abundantly found during the assays, especially at acid pH where the radical pathway is disadvantaged, as shown in Fig. 4. Even though the pattern of fragmentation of this compound could not be entirely interpreted, the presence of the ion fragment at m/z 122 ($C_6H_4NO_2$) with a DBE (double bond equivalency) of 6, confirms the presence of the nitrobenzene moiety in the molecule.

Compound TP-284b was assigned to the oxidation of the methyl group at the isoxazole ring to yield a carboxylic acid functional group. The fragmentation pattern confirmed the proposed structure (Fig. 3) showing typical losses of −18 uma (m/z 202) and −28 uma (m/z 174), which corresponded to the neutral losses of $-H_2O$ and –CO characteristic from the carboxylic moiety. The mass spectrum of the last of the isobaric compounds identified (TP-284a)

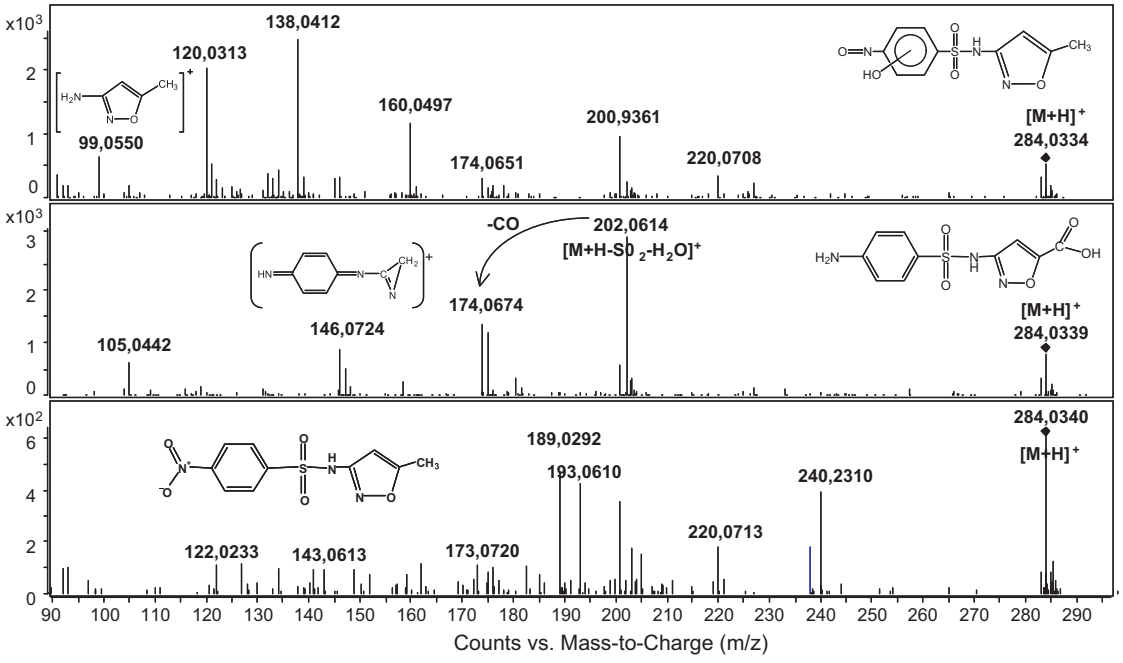


Fig. 3. Product ion spectra of three isobaric compounds identified during ozonation processes.

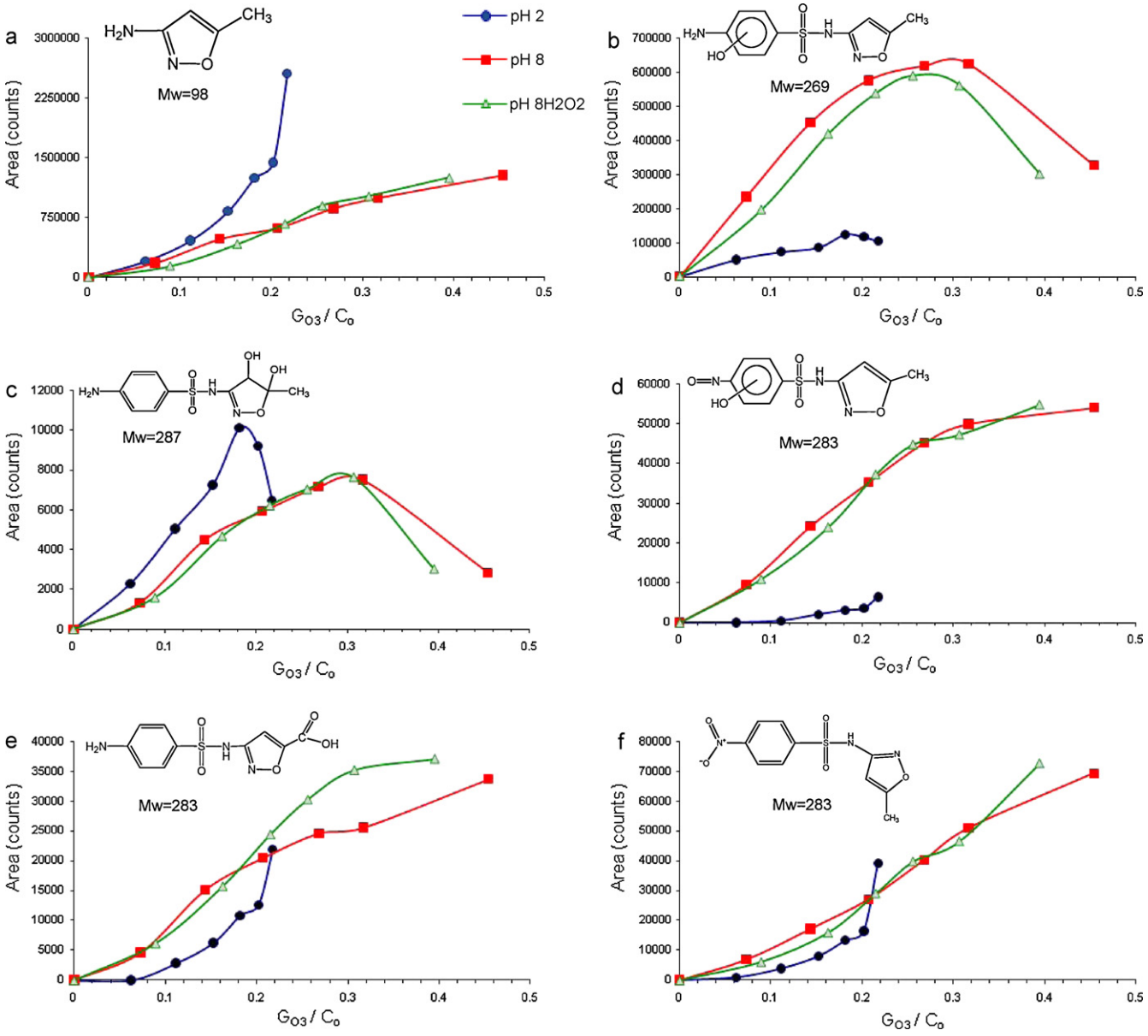


Fig. 4. Formation kinetics of the TPs identified during ozonation at pH 2 (●), pH 8 (■) and pH 8/H_2O_2 (▲).

showed the ion at *m*/*z* 99.0550, corresponding to the isoxazole moiety, and absence of ions at *m*/*z* 108, 156 or 160, typical of the p-sulfoaniline ring. This proved again that the oxidation took place in the p-sulfoaniline ring, thus yielding the structure set out in Fig. 3.

The intermediates identified evidenced the occurrence of various degradation reactions, as it is shown in Fig. 5, which included: (i) hydroxylation of the benzene ring (TP-270); (ii) oxidation of the amine group at the benzene ring (TP-284a and TP-284c); (iii) oxidation of the methyl group at the isoxazole ring (TP-284b); (iv) oxidation of the double bond C_{11}=C_{12} at the isoxazole ring (TP-288); and (v) S–N bond cleavage (TP-99). These reactions took place during the ozonation of SMX in all the experimental conditions assayed (pH 2, pH 8 and pH 8/H_2O_2), suggesting that SMX is removed by the same paths, but the dominance of one over the other depended on such conditions. Thus, although all the intermediates were present at the three ozonation runs, their generation rates were different, as shown in Fig. 4.

In general, at pH 8 and pH 8/H_2O_2 all the TPs showed very similar behaviour in terms of abundance and formation rates. Greater differences were observed, however, with experiments performed at acidic pH. This is consistent with the different mechanisms governing the oxidation process. Thus, hydroxylation reactions involving the attack of hydroxyl radicals at the benzene ring (TP-270, TP-284a) were mainly promoted at basic pH when the formation of these radicals is enhanced. Fewer differences were observed between the formation rates of TP-284b and TP-284c at pH 8 and pH 2, thus confirming the functional groups –NH_2 and –CH_3, at the benzene and isoxazole ring to be the preferred sites attacked by ozone [30]. Oxidation of the amine group to yield the nitro-SMX derivative has been reported only during SMX ozonation. The cleavage of sulphonamide bond is the main degradation pathway, and it also generates the most abundant intermediate (TP-99). The formation rate of this compound is higher at pH 2 than at pH 8, when direct ozonation is favoured. The formation of 4-aminobenzene sulphonamide as reported by Rodayan et al. [31] could not be observed under the operational conditions assayed.

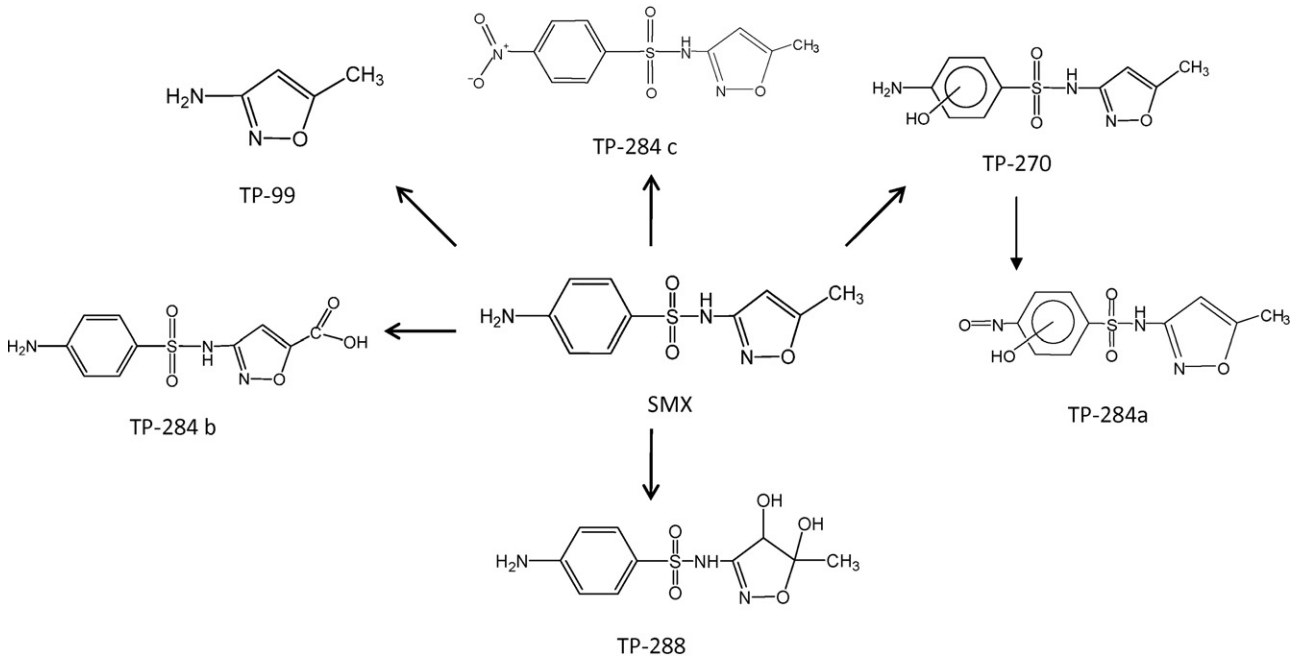


Fig. 5. Degradation pathways of SMX, which include: (1) hydroxylation of the benzene ring; (2) oxidation of the amine group at the benzene ring; (3) oxidation of the methyl group at the isoxazole ring; (4) oxidation of the double bond C=C at the isoxazole ring; and (5) S–N bond cleavage.

3.2. Toxicity of reaction mixtures

The toxicity of reaction mixtures was assessed using a *D. magna* acute immobilization test and a *P. subcapitata* multigenerational chronic growth inhibition bioassay. For a solution containing 29.5 ± 0.5 mg/L of SMX, we obtained a 48 h immobilization for *D. magna* of 68.3 ± 3.1%, close to EC_{50} value and consistent with the median effect value reported in the literature for 25.2 mg/L [32]. The observed toxicity for non-treated sulfamethoxazole also agreed with data previously published [33]. The results of toxicity bioassays applied to samples taken during the runs at different reaction times are shown in Fig. 6 in which the error bars represent 95% confidence intervals. The different results obtained for *D. magna* at pH 2 and 8 (Fig. 6a) probably reflect the different rates of SMX removal. In fact, SMX disappeared from solution for an ozone dose of 0.2 mM, at which there was a similar value for the immobilization of *D. magna* of about 50% at both pH 2 and 8. The results corresponding to the growth inhibition of *P. subcapitata*, shown in Fig. 6b, indicate a considerable increase in toxicity for doses up to the 0.02–0.05 mM range. Although the growth inhibition was reduced thereafter, it still reflected toxicities twice as high as those corresponding to untreated SMX for doses as high as 0.2 mM, at which there is no remaining SMX in solution. The bioassays clearly show that considerable toxicity exists in ozonated samples even after the complete depletion of SMX; this toxicity can only be attributed to oxidation by-products. Moreover, for intermediate ozone dosing, the first oxidation products originate a considerable increase in toxicity which, in the case of *P. subcapitata*, leads to a growth inhibition of over 90% at pH 8. A toxicity increase in treated samples due to the formation of by-products that can cause greater toxicity than the parent compound has been observed previously [8,9,34]. Shang et al. [34] reported a toxicity increase during the early stages of the ozonation of chlorophenols that could be attributed to the formation of chlorocatechols, chloromuconic acids and other hydroxylated or chlorinated compounds. Rosal et al. [9] identified the formation of oxidation products during the ozonation of clofibric acid, particularly hydroquinone and 4-chlorophenol. Fig. 4 shows the time evolution of the areas corresponding to the chromatographic peaks

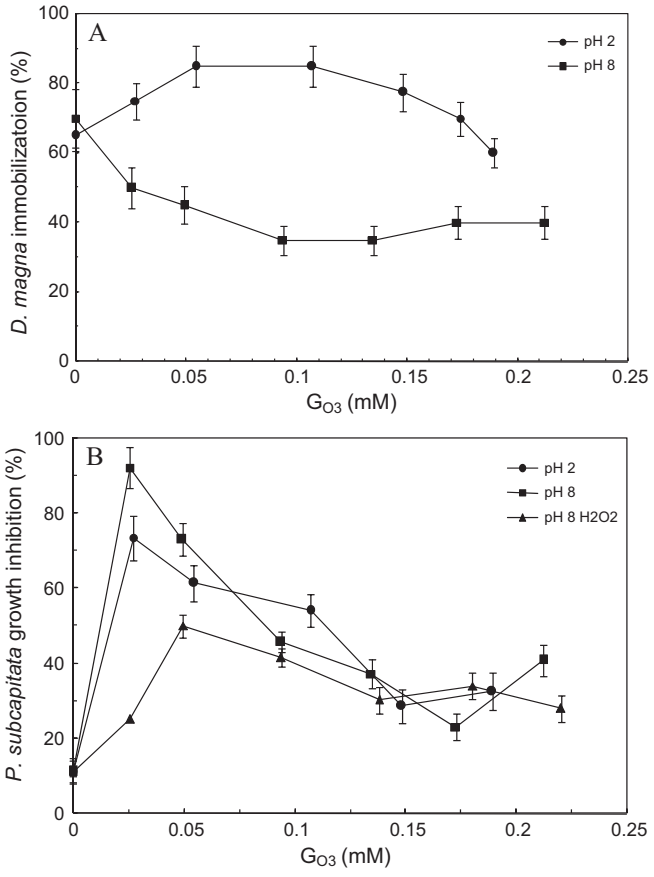


Fig. 6. Toxicity assessed by immobilization of *D. magna* (A, 48 h) and growth inhibition of *P. subcapitata* (B, 72 h) as a function of the amount of ozone transferred to the liquid phase for runs pH 2 (●), pH 8 (■) and pH 8/H_2O_2 (▲).

of TPs 99, 270, 288, 284a, 284b and 284c for ozone doses up to 0.12 mM for which the conversion of SMX was between 65% (pH 2) and 90% (pH 8/H_2O_2). At the light of the results, it is difficult to assign the toxicity to a specific intermediate. Since the toxic effect increase at the early stages of the treatment and then decrease at high ozone dosing, it could be associated with those intermediates that exhibit a similar behaviour, such as the hydroxylated derivatives TP-270 or TP-288. However, derivatives of 4-aminobenzenesulfonic acid, not detected in this work, or other minor compounds might be involved in the observed increase in toxicity.

4. Conclusions

The ozonation of sulfamethoxazole in aqueous solution was performed in fast kinetic regime, which provides a low availability of ozone in water. This operational mode optimizes ozone consumption, minimizing the ozone concentration in water. Irrespective of pH, the complete removal of SMX took place for ozone doses lower than 0.2 mM and was faster under operational conditions favouring the generation of radical species (pH 8 and pH 8/H_2O_2). The analytical evaluation of reaction mixtures enabled us to identify the reaction intermediates from the direct attack of molecular ozone and indirect reaction with ozone-generated hydroxyl radicals. The formation of oxidation by-products is unavoidable because the optimization of the ozone consumption for total mineralization would lead to long reaction times and economically unviable processes. The main degradation routes identified in this work are the hydroxylation of the benzene ring, the oxidation of the amino group on the benzene ring, the oxidation of the methyl group in the isoxazole ring and the double bond of the isoxazole ring and S–N bond cleavage. Six ozonation by-products have been identified by LC-ESI-QTOF-MS, the most abundant of which was the product of S–N bond cleavage. The toxicity of partially ozonated samples for *D. magna* and *P. subcapitata* revealed the formation of toxic by-products during the early stages of reaction when there was still SMX in solution, and the persistence of an important residual toxicity for *P. subcapitata* after the total depletion of sulfamethoxazole. This work emphasizes the need for a toxicological characterization of ozonated samples as the removal of parent pollutants would not be enough to avoid the release of toxic species in the environment.

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