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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