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ORIGINAL ARTICLE

Residues of selected antibiotics in the South Moravian Rivers, Czech Republic

Katerina Jarova [1], Milada Vavrova [2], Alice Koleckarova [1]

1 Department of Ecology and Diseases of Game, Fish and Bees, Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic
2 Institute of Chemistry and Technology of Environmental Protection, Faculty of Chemistry, Brno University of Technology, Czech Republic

Correspondence to: Mgr. Katerina Jarova, PhD.
Department of Ecology and Diseases of Game, Fish and Bees
Faculty of Veterinary Hygiene and Ecology
University of Veterinary and Pharmaceutical Sciences Brno
Palackeho tr. 1/3, 612 42 Brno, Czech Republic.
TEL: +420541562656; E-MAIL: jarovak@vfu.cz

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Abstract

OBJECTIVES: The aim of this study was to assess the contamination level of aquatic ecosystems of the Oslava and the Jihlava Rivers, and of the Nove Mlyny Water Reservoir, situated in the South Moravian Region (Czech Republic), by residues of selected veterinary pharmaceuticals. We isolated and determined 10 sulfonamide antibiotics in samples of surface water and bottom sediments using optimized analytical methods.

DESIGN: A representative number of sampling sites in the entire basin of selected waters were chosen. Samples were collected particularly near the larger cities in order to assess their possible impact to the aquatic ecosystems. Extraction, pre-concentration and purification of samples were performed using optimized methods of solid phase extraction and pressurized solvent extraction. Final identification and quantification were carried out by high-performance liquid chromatography coupled with diode array detector.

RESULTS: The concentration of sulfonamides in water samples were all under the limit of detection. Regarding sediment samples, sulfadimidine was found at most sampling sites; its highest values were recorded in the Jihlava River (up to 979.8 $\mu g.kg^{-1}$ dry matter). Other frequently detected sulfonamides were sulfamethoxazole and sulfamerazine. Most other sulfonamides were under the limit of detection or limit of quantification.

CONCLUSIONS: Monitoring of antibiotic residues in the environment, especially in the aquatic ecosystem, is a current topic due to the growing worldwide use in both human and veterinary medicine. According to obtained results, we document the pollution of selected rivers and water reservoir by particular sulfonamides which basically reflects their application in veterinary medicine.

Abbreviations

DAD	- diode array detector
DI	- deionized
DM	- dry matter
HLB	- hydrophilic-lipophilic balance
HPLC	- high performance liquid chromatography
LC/MS	- liquid chromatography/mass spectrometry
LOD	- limit of detection
LOQ	- limit of quantification
ND	- not detected
PEP	- polymeric SPE sorbent made of polydivinylbenzene modified by vinyl prolidon
POPs	- persistent organic pollutants
PSE	- pressurized solvent extraction
R2	- coefficient of determination expressing the reliability
RSD	- relative standard deviation
SCL	- sulfaclozine
SDIA	- sulfadiazine
SDM	- sulfadimidine (sulfamethazine)
SDMT	- sulfadimethoxine
SDX	- sulfadoxine
SGN	- sulfaguanidine
SMR	- sulfamerazine
SMX	- sulfamethoxazole
SPE	- solid phase extraction
SPY	- sulfapyridine
STZ	- sulfathiazole
SÚKL	- State Institute for Drug Control
ÚSKVBL	- Institute for State Control of Veterinary Biologicals and Medicines
WR	- water reservoir
WWTP	- wastewater treatment plant

INTRODUCTION

Antibiotics, including sulfonamides, belong to a large group of pharmaceuticals, consumption of which continues to grow in both human and veterinary medicine. Contamination of the environment by pharmaceuticals has received increasing amounts of attention in recent years because these compounds have been continually released into the aquatic environment in huge quantities (Daughton & Ternes 1999; Plhalova *et al.* 2014). The occurrence and fate of pharmaceutically active compounds has been recognized as one of the emerging issues in environmental chemistry (Heberer 2002). In both humans and animals, antibiotics are metabolized and excreted in urine or feces, while up to 30–90% of them can be excreted in active form. In this way, antibiotics in their original or metabolized form can enter the environment where they can accumulate and persist. As a result, these compounds can negatively affect ecosystems, including water ecosystems, where they are toxic especially to non-target aquatic organisms. Veterinary antibiotics are often more hazardous for the environment than those used to treat people. Animals consume up to 60% of antibiotics, not only for veterinary treatment but also as a feed supplement for disease prevention. The occurrence of residual antibiotics in the environment then results in an increased incidence of resistant bacteria, which may become a potential threat to human health in the future (Boxall *et al.* 2002; Heberer 2002; Costanzo *et al.* 2005; Cooper *et al.* 2008; Watkinson *et al.* 2009; Plhalova *et al.* 2014).

One of the most important sources of pharmaceuticals in the aquatic environment are medicines excreted from the bodies of humans that enter the water ecosystem after going through the wastewater treatment plant (WWTP) (Heberer 2002; Kotyza *et al.* 2009; Chen *et al.* 2012). The issue of efficiency of WWTPs for removing drug residues or other persistent organic pollutants (POPs) is subject to many studies worldwide (Costanzo *et al.* 2005; Babic *et al.* 2006; Cooper *et al.* 2008; Diaz-Cruz *et al.* 2008; Kummerer 2009; Watkinson *et al.* 2009; Xu *et al.* 2012; Milic *et al.* 2013; Zelnickova *et al.* 2013). Another significant source of contamination by pharmaceuticals in the environment can be due to improper disposal of medications. These substances may then enter surface waters either through leaking from landfills or by flushing down the toilet (Heberer 2002; Kotyza *et al.* 2009). Another important source of drug residues in the environment is represented by veterinary pharmaceuticals that can get into surface or ground water through the use of livestock excrement for fertilization or through sludge from a WWTP, which is often used as an additional fertilizer in agriculture (Daughton & Ternes 1999; Boxall *et al.* 2002; Heberer 2002; Kotyza *et al.* 2009; Li *et al.* 2011; Chen *et al.* 2012). Finally, industrial multi-stand animal farms are responsible for some areas of locally high concentrations of pharmaceuticals (Piotrowicz-Cieslak *et al.* 2012).

The aim of this study was to assess the contamination of significant rivers and water reservoir of the Morava River Basin and South Moravian Region by residues of selected antibiotics used especially in veterinary medicine, as follows from the data about their use and consumption in the Czech Republic, obtained from the Institute for State Control of Veterinary Biologicals and Medicines (ÚSKVBL) and from the State Institute for Drug Control (SÚKL).

MATERIAL AND METHODS

Chemicals and materials

All organic solvents, sorbents and other chemicals used for the analysis were of high purity for the residual analysis. Methanol for gradient elution was purchased either from Sigma-Aldrich (Steinheim, Germany) or LC-MS quality from Biosolve (Valkenswaard, The Netherlands). Acetonitrile gradient grade for liquid chromatography, deionized water for chromatography and formic acid (98–100%) were purchased from Merck (Darmstadt, Germany). High-purity nitrogen (99.996%) was supplied by Messer Technogas (Prague, Czech Republic). Analytical standards of sulfonamide antibiotics (sulfaguanidine, sulfadiazine, sulfathiazole, sulfapyridine, sulfamerazine, sulfadimidine, sulfamethoxazole, sulfaclozine, sulfadimethoxine) were purchased from Sigma-Aldrich, Fluka (Steinheim, Germany); sulfadoxine from Dr. Ehrenstorfer (Augsburg, Germany).

Sampling

Samples of surface water and bottom sediments were taken from the Oslava River (8 sampling sites) and from the Jihlava River (7 sampling sites) which are two main rivers flowing through the South Moravian Region of the Czech Republic (together with the Svratka and the Svitava Rivers that are the subject of another study – Jarova *et al.* 2014). Samples from 6 sampling sites were also taken from the Nove Mlyny Water Reservoir, into which the two rivers (after their mutual confluence) estuary. These sites were selected and monitored because of their location upstream and downstream to some significant possible source of contamination by residues of antibiotics. Distribution of the sampling sites along the entire length of rivers allowed a comprehensive assessment of the water ecosystem burden by selected drugs. Altogether a total of 21 samples from each matrix were collected, while there were always pooled samples of three individual samples from one sampling site. The water samples were collected in clean 1 L amber glass bottles; the sediment samples were collected in clean 1 kg plastic container. The map of all sampling sites is shown in Figure 1. Sampling was carried out in August and September 2013. All samples were processed immediately after their delivery to the laboratory or kept refrigerated until the next day.

Pre-analytical procedures

The target analytes were isolated from samples of water and sediment using optimized methods. The efficiency of methods were verified using analytical standards of selected sulfonamides in concentration of 25 $\mu g.mL^{-1}$ for water and 50 $\mu g.mL^{-1}$ for sediment (solved in 0.1 $mol.L^{-1}$ formic acid/methanol). As analytically pure matrices, free of all analytes, DI water and sea sand (Lachema – Neratovice, Czech Republic) were used for optimization.

All water samples were first filtered using a vacuum pump through a glass-microfiber filter to remove coarse particles. Pre-concentration and purification of the sample, as well as extraction of monitored sulfonamides were carried out by solid phase extraction (SPE). The Supel-Select HLB cartridges (200 mg/6 mL) from Supelco (Bellefonte, PA, USA) were used. The conditions of SPE were as follows: conditioning with 2 mL of methanol and 2 mL of DI water; application of the sample (500 mL); washing the sorbent with 2 mL of 5% acetonitrile; drying under vacuum for 10 min and analytes elution with 5 mL of methanol:acetonitrile (1:1).

For sediment samples, the percentage of dry matter was determined first. Remaining sediment was dried out at a temperature of 105 °C into the constant weight and homogenized. The method of pressurized solvent extraction (PSE), using a combination of high pressure and high temperature, was used for extraction of monitored sulfonamides from dried sediment samples. A total of 15 g of each sample were mixed with 3 g of hydromatrix (Applied Separations – Allentown, PA, USA) and transferred into special PSE vessel. The conditions of PSE were as follows: temperature 40 °C; pressure 6 MPa; static phase 7 min; nitrogen drying 2 min; solvent flushing 20 s; 2 cycles, methanol. Due to the SPE as a next step, it was essential to turn the extract into water phase. Therefore, methanol was evaporated to dryness and sample was re-dissolved in 50 mL of 10% methanol using ultrasonic bath. Purification and preconcentration of sediment extracts were carried out by SPE, while Cleanert PEP-Plus cartridges (500 mg/6 mL) from GS-Tek (Newark, DE, USA) were used. The conditions of SPE were as follows: conditioning with 2 mL of 0.1 $mol.L^{-1}$ formic acid/methanol and 2 mL of 5% methanol; application of the sample (50 mL); washing the sorbent with 2 mL of 5% methanol; drying under vacuum for 10 min and analytes elution with 4 mL of 0.1 $mol.L^{-1}$ formic acid/methanol.

All eluates were evaporated to dryness, re-dissolved in 1 mL of 0.1 $mol.L^{-1}$ formic acid/methanol and transferred through nylon filters (0.45 µm; Labicom – Olomouc, Czech Republic) into vials. Each sample was performed in two parallel determinations.

Analytical determination

Final identification and quantification of selected analytes were carried out by high performance liquid chromatography coupled with diode array detector (HPLC/DAD) using Agilent 1100 Series device (Agilent Technologies – Santa Clara, CA, USA). For the chromatographic separation ZORBAX Eclipse XDB-C8 column (2.1 × 150 mm; 3.5 µm) was used. Following conditions were applied: injection volume 1 µL; flow rate 0.250 $mL.min^{-1}$; column temperature 35 °C; detection wavelength 270 nm. Two mobile phases, 0.01 $mol.L^{-1}$ formic acid in MilliQ-water and methanol, were used in gradient (Table 1). The total time of one analysis was 30 minutes at these conditions. All data were processed by means of the ChemStation software (Agilent Technologies – Santa Clara, CA, USA).

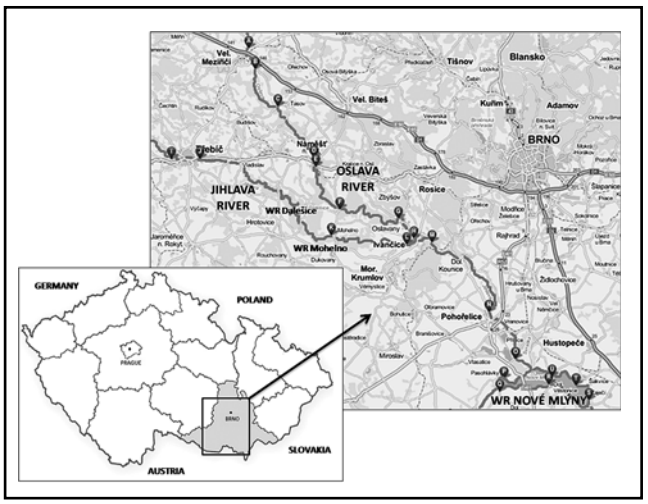

Fig. 1. Map of the Czech Republic with the sampling area and sampling sites.

Tab.1. The HPLC gradient of mobile phases.

Time [min]	Methanol [%]	0.01 mol.L^{-1} formic acid [%]
3.0	10.0	90.0
4.0	30.0	70.0
11.0	30.0	70.0
15.0	20.0	80.0
20.0	40.0	60.0
20.5	70.0	30.0
25.0	70.0	30.0
27.0	10.0	90.0

Tab. 2. Monitored sulfonamides and their abbreviations, coefficients of determination (R^2), limits of detection (LOD) and limits of quantification (LOQ).

Sulfonamide		R^2	LOD [μg.mL^{-1}]	LOQ [μg.mL^{-1}]
sulfaguanidine	SGN	0.9999	0.081	0.271
sulfadiazine	SDIA	0.9997	0.104	0.347
sulfathiazole	STZ	1.0000	0.163	0.542
sulfapyridine	SPY	0.9998	0.116	0.387
sulfamerazine	SMR	0.9999	0.054	0.178
sulfadimidine	SDM	0.9999	0.057	0.191
sulfamethoxazole	SMX	0.9999	0.069	0.230
sulfadoxine	SDX	0.9998	0.083	0.277
sulfaclozine	SCL	0.9999	0.214	0.713
sulfadimethoxine	SDMT	0.9999	0.432	1.442

Methodology optimization

Both extraction methods together with analytical determination were optimized using analytical standards of selected sulfonamides and spiked matrices. Mean values of their recoveries were 79.0% for the analytical procedure applied to water samples, and 78.8% for the analytical procedure of sediment samples. Calibration lines from standard stock solutions ranging in concentrations of 1–100 μg.mL^{-1} were constructed for each of the observed sulfonamide while the coefficient of determination value (R^2) was always higher than 0.9997 (Table 2). Repeatability expressing the precision and accuracy of the optimized analytical method was calculated by measuring of five model samples spiked with the standard, and its values are expressed by the relative standard deviation (RSD). Repeatability of the method for determination of water samples ranged from 1 to 7%, depending on individual analyzed drug. For the method to determine sediment samples, repeatability was calculated in the range of 5–7%. For each sulfonamide antibiotic the limit of detection (LOD) and the limit of quantification (LOQ) were calculated by Eq. (1) and (2), respectively (see Table 2), using the lowest point of the calibration and the average height of 15 peaks of noise over the entire length of the real sample base line. *S* in the equation represents the height of the analyte signal; *N* is the height of the noise signal.

$$\text{LOD } [\mu g.mL^{-1}] = 3 \times \left(\frac{c\ [\mu g.mL^{-1}]}{S/N}\right) \quad (1)$$

$$\text{LOQ } [\mu g.mL^{-1}] = 10 \times \left(\frac{c\ [\mu g.mL^{-1}]}{S/N}\right) \quad (2)$$

RESULTS AND DISCUSSION

Based on this study, the residues of sulfonamide antibiotics in water samples from the Oslava and Jihlava Rivers, as well as from the Nove Mlyny Water Reservoir, were either not detected at all (ND) or their concentration was under the limit of detection (<LOD), which ranges from 0.054 to 0.432 μg.mL^{-1} for individual sulfonamides (Table 2). According to the literature, the solubility of individual sulfonamides in water varies (Babic *et al.* 2006; Viteckova *et al.* 2008; Milic *et al.* 2013). Sulfonamides are substances of amphoteric quality and thus their pH value ranges into both acidic and basic areas (from 4.5 to 9). Due to the N-H chemical bond in the sulfonamide group, sulfonamides behave more as weak acids, which are polar and generally soluble in water and polar solvents. However, the solubility is highly pH-dependent and that is why it often differs (Diaz-Cruz *et al.* 2005, 2008; Babic *et al.* 2006; Lincova & Farghali 2007). This fact can explain why there is a very low concentration of sulfonamide residues recovered from surface waters. A similar trend of very low concentration or no detection of sulfonamide antibiotics in surface water was also observed in various studies (Lindsey *et al.* 2001; Kolpin *et al.* 2002, 2004; Batt *et al.* 2006; Diaz-Cruz *et al.* 2008; Watkinson *et al.* 2009; Lacina *et al.* 2012; Jarova *et al.* 2014).

Altogether 21 samples from each matrix were collected from the previously referred rivers and water reservoir, with exception of one sampling site at Nove Mlyny (T-20) where it was impossible to collect the sediment because of the rocky bottom. Concerning the other 20 sediment samples, the majority of monitored sulfonamides were under the limit of detection (<LOD), limit of quantification (<LOQ) or were not detected at all (Table 2). Sulfonamides detected in sediment samples in measurable concentrations were sulfamerazine (SMR), sulfamethoxazole (SMX) and sulfadimidine (SDM). Residues of SMR occurred at only one sampling site (concentration 10.59 μg.kg^{-1} DM), which was the first sampling site at the Jihlava River, located upstream from the Trebic City (sample I-9). Additionally, this site was significantly burdened with other sulfonamides in relatively high concentrations, which could be explained as point source pollution. SMX was detected five times altogether in the entire sampling area, twice in sediment from the Oslava River and three times in the Jihlava River, usually at low concentrations

(ranging from 11.22 to 14.90 μg.kg^{-1} DM). An exceptionally high concentration of SMX (90.73 μg.kg^{-1} DM) was detected at the above-mentioned sampling site in the Jihlava River (I-9). However, neither SMR nor SMX were detected at any of the sampling sites located at the Nove Mlyny WR. Both sulfonamides (SMR and SMX), particularly sulfamethoxazole, are often used in human and veterinary medicine in combination with trimethoprim (e.g. Co-trimoxazole) for treatment of urinary tract infections, ear inflammation, bronchitis or other respiratory tract infections (Papich 2010; Hauser 2012). Sulfonamide sulfadimidine (SDM), also known as sulfamethazine in the literature, was detected in almost all sediment samples taken from the sampling area of the South Moravian Region. Concentration values of SDM ranged from 7.808 (Nove Mlyny) to 979.8 (Jihlava) μg,kg^{-1} DM; specifically 11.67–242.3 μg.kg^{-1} DM at the Oslava River sampling sites, 9.513–979.8 μg.kg^{-1} DM at the Jihlava River sampling sites, and 7.808–119.5 μg.kg^{-1} DM in sediment samples from the Nove Mlyny WR. Levels of SDM concentrations in sediment samples from the entire sampling area are compared in Figure 2. Sites with the greatest amount of contamination were generally located close to larger cities (Namest nad Oslavou, Oslavany, Ivancice, Trebic etc.). As known from the literature, large cities and agglomerations are the main source of pharmaceutical residues in the aquatic ecosystem (Pei *et al.* 2006; Kotyza *et al.* 2009; Watkinson *et al.* 2009; Chen *et al.* 2012), as the technologies used originally in WWTPs may not have been effective enough (Babic *et al.* 2006; Kotyza *et al.* 2009). Many WWTPs in the South Moravian Region of the Czech Republic are currently being built, either new-built or under reconstruction (project of EU until 2015). Another important source of contamination of aquatic ecosystems by antibiotics may be hospitals in the above-mentioned cities. Possible explanation of the fact that higher contaminated sites were often situated upstream the monitored towns compare to those located downstream could be significant dilution of the river by purified water from WWTPs, which would indicate their good cleaning efficiency against the monitored drugs in this area. There can be also difference in various structures of the bottom sediment as stones, gravel, sand, mud, etc., where the residues of pharmaceuticals are able to adhere and persist less or more. Figure 3 shows the overall contamination of sediment samples from monitored rivers and water reservoir by sulfonamide antibiotics, which is expressed as the sum of all 10 selected sulfonamides. Most concentrations of selected sulfonamides in sediment samples from this study were comparable to values published in the literature, extremely high concentrations of sulfadimidine at some sampling sites were however much higher than found in the literature (Pei *et al.* 2006; Bai *et al.* 2014; Muziasari *et al.* 2014). However, studies on contamination of sediments by sulfonamide antibiotics are not published often.

In conclusion, this study assessed the contamination level of aquatic ecosystems of the Oslava and the Jihlava Rivers, and of the Nove Mlyny Water Reservoir, situated in the South Moravian region of the Czech Republic, by determining residues of 10 sulfonamide antibiotics, which are being often used in human and veterinary medicine especially. There were detected very low (<LOQ) or even non-detectable (<LOD) concentrations of sulfonamides in water samples and in the majority of sediment samples. The most frequently

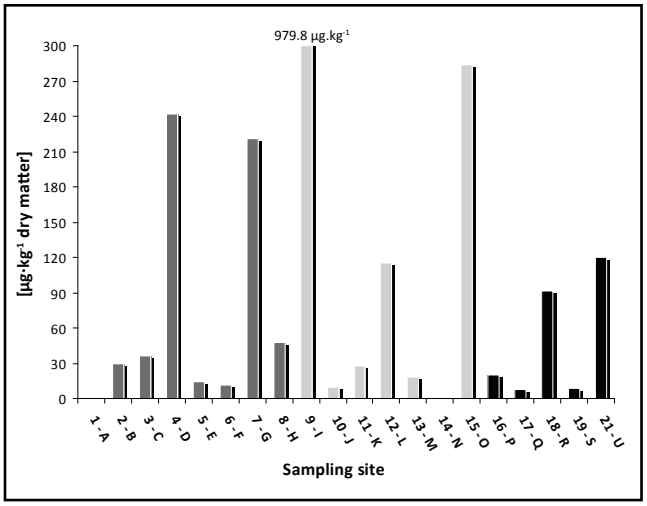

Fig. 2. Comparison of concentrations of sulfadimidine residues detected in sediment samples from Oslava River (1A–8H), Jihlava River (9I–15O) and Nove Mlyny Water Reservoir (16P–21U).

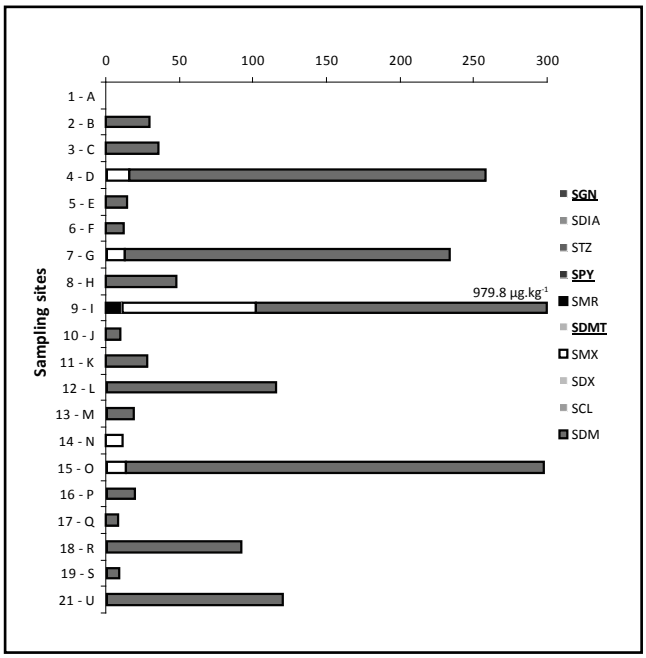

Fig. 3. Content of sulfonamide antibiotics in sediment samples expressed as the total contamination [Σ10 sulfonamides in μg. kg^{-1} DM]. SGN – sulfaguanidine; SDIA – sulfadiazine; STZ – sulfathiazole; SPY – sulfapyridine; SMR – sulfamerazine; SDMT – sulfadimethoxine; SMX – sulfamethoxazole; SDX –sulfadoxine; SCL – sulfaclozine; SDM – sulfadimidine

detected sulfonamide, in relatively high concentration, was sulfadimidine (SDM), followed by sulfamethoxazole (SMX) and sulfamerazine (SMR). The occurrence of particular sulfonamide antibiotics in the aquatic environment basically reflects their application in human and veterinary medicine. According to this and other related studies, it is important to note that water ecosystems can be contaminated with the residues of antibiotics, and therefore monitoring of these hazardous pollutants is at a high priority.

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