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Occurrence of veterinary antibiotics and chemotherapeutics in fresh water, sediment, and fish of the rivers and lakes in Poland

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Abstract

The occurrence of commonly used veterinary antimicrobial agents was investigated in 159 fresh water, 443 fish, and 150 sediment samples from Polish rivers and lakes. The agents included aminoglycosides, β-lactams, diaminopyrimidines, fluoroquinolones, lincosamides, macrolides, pleuromutilins, sulfonamides, and tetracyclines. The analysis was performed by three different sample preparation procedures for each matrix and it was performed by liquid chromatography-tandem mass spectrometry with electrospray ionisation source in positive mode, under the same conditions. All analytical methods used were validated and showed good sensitivity, accuracy, and precision. The LOQ was in the range from 5 µg/kg to 125 µg/kg for fish samples, from 0.02 µg/L to 10 µg/L for fresh water samples, and from 1 µg/kg to 8 µg/kg for sediment samples.

Keywords: antibiotics, water, sediments, fish, LC-MS/MS, Poland.

Introduction

Antimicrobial compounds are widely used in human and veterinary medicine to protect human and animal health, to prevent economic losses, and to help to ensure a safe food supply. After administration, the antibiotics may pass through the sewage system and end up in the environment, mainly in the water compartment. There are many ways for antibiotics to be transferred to water. Human pharmaceuticals discharge into the environment mainly through sewage treatment plants. Veterinary pharmaceuticals can spread to the water environment through direct application in aquaculture, wash off from topical treatments livestock treatment plants, and from manure-treated farmlands (7). Residual amounts of still-active antibiotics can reach surface water, sediments, and aquatic animals. This situation can cause the ecological contamination, unintended antibiotic passage into organisms, and promotion of dissemination of antibiotic resistant bacteria and resistance genes among bacterial populations. Once released to the environment, antibiotics may run into the aquatic system and affect the ecosystem, what can have a negative influence on human health (7, 8). Several publications have reported the occurrence of various veterinary and human pharmaceuticals, including antibiotics, in surface water, groundwater, wastewater, sediments, and soil. Most of the publications reported the occurrence of sulfonamides (1, 2, 4-6, 9-11, 13, 15-17), fluoroquinolones (2, 4, 5, 10, 15, 17), tetracyclines (4, 7-10, 13, 17), and macrolides (2, 4, 7, 8, 13, 15, 16) in samples from different sources within the aquatic environment.

Because there is not much information on the presence of veterinary antibacterial agents in Polish rivers and lakes, it was decided to study the occurrence of various commonly used veterinary antibacterial compounds in Polish rivers and lakes. The material was collected away from urban areas. Six rivers (Vistula, Warta, Oder, Brda, Wkra, and Dunajec) and three lakes (Lanskie, Maroz, and Rybnik power station reservoir) were selected for the contaminant occurrence study, specifically for veterinary antibiotics in fresh water, sediment, and fish samples. The antibiotics were determined with optimised and validated analytical methods by liquid chromatography-tandem mass spectrometry.

Material and Methods

Reagents. All reagents were of an analytical grade. Citric acid, sodium acetate, ethylenediaminetetraacetic acid (EDTA), and sodium hydroxide were from (POCH) (Poland), meta-phosphoric acid, acetonitrile and methanol were obtained from J.T. Baker (the Netherlands). Heptafluorobutyric acid (HFBA) was from Fluka, (USA). Trichloroacetic acid (TCA) was from Sigma-Aldrich, (USA). Water was deionised (>18 $M\Omega cm^{-1}$) by the Millipore system. The: amoxicillin (AMOX), ampicillin (AMPI), penicillin G (PEN G), nafcillin (NAF), dicloxacillin (DICLOX), oxacillin (OXA), cephapirin (CFPI), ceftiofur (CFT), cefoperazone (CFPE), cephalexin (CFLE), cefquinome (CFQ), cefazolin (CFZ), cefalonium (CFLO), danofloxacin (DAN), difloxacin (DIF), enrofloxacin (ENR), ciprofloxacin (CIP), norfloxacin (NOR), marbofloxacin (MAR), flumequine (FLU), sarafloxacin (SAR), oxolinic acid (OXO), nalidix acid (NAL), chlortetracycline (CTC), tetracycline (TC), doxycycline (DC), oxytetracycline (OTC), streptomycin (STRP), dihydrostrepromycin (DISTRP), spectinomycin (SPEC), neomycin (NEO), sulfamerazine (SME), sulfamethazine (SMT), sulfamethoxazole (SMA), sulfamonomethoxine (SMM), sulfadimethoxine (SDMX), sulfathiazole (SFT), trimethoprim (TMP), tylosin (TYL), erythromycin (ERY), spiramycin (SPI), tilmicosin (TIL), josamycin (JOS), lincomycin (LIN), tiamuline (TIM), and sulfafenazole (SFF) – internal standard (IS), were from Sigma-Aldrich (USA). Strata X (100 mg, 6 mL) cartridges were obtained from Phenomenex (USA), Oasis HLB (60 mg, 3 mL) cartridges were obtained from Waters (USA), and the 0.22 μm PVDF syringe filters were from Restek (USA).

Analytical standards and standard solutions. Individual stock standard solutions (1 mg/mL) for tetracyclines (TC, CTC, DC, OTC), macrolides (TYL, ERY, SPI, TIL, JOS), sulfonamides (SME, SMT, SMA, SMM, SDMX, SFT), diaminopyrimidines (TIM), and lincozamides (LIN) were prepared in methanol in amber volumetric flasks and stored at -18°C. For aminoglycosides (STRP, DISTRP, SPEC, NEO) and β-lactams (AMOX, AMPI, OXA, DIKLOX, PEN G, NAF, CFPI, CFT, CFLE, CFQ, CFZ), standard solutions were prepared in deionised water in amber volumetric flasks and stored at -18°C. Cefalosporines (CFLO and CFPE) were prepared in acetonitrile and water (1:1, v/v) in amber volumetric flasks and stored at -18°C. Whereas for fluoroquinolones (DAN, DIF, ENR, CIP, NOR, FLU, SAR, OXO, NAL, MAR) standard solutions were prepared in methanol with addition of sodium and stored in amber volumetric flasks at -18°C. Mixtures of working standard solutions were prepared in deionised water in plastic flasks and stored at 4°C. The individual stock internal standard (IS) solution (2 μg/mL) for SFF was prepared in deionised water in amber volumetric flasks and stored at -18°C. The working internal standard solution (2 μg/mL) was prepared in deionised water in amber volumetric flasks and stored at 4°C.

Sample collection. In spring and autumn 2011, 41 surface water, 44 sediment, and 240 fish samples were collected and analysed. 174 fish, 98 water, and 86 sediment samples were collected and analysed in 2012. In spring 2013, only 29 fish, 20 water, and 20 sediment samples were collected and analysed. The sampling locations are shown in Fig. 1. Fresh water, sediment, and fish samples were collected from 14 sampling points (Polish rivers and lakes). A few of them were located near large urban areas (Cracow, Wroclaw, and Gorzow Wielkopolski) but away from wastewater points. Some sampling points were located near livestock farms (Brda river and Wkra river) to check the impact of large clusters of such farms. Other sample collecting points were located in protected areas like the Maroz lake and Dunajec river. Also, a number of samples was collected from industrial areas like Rybnik power station water reservoir. Such contrasting locations of sampling points were chosen to reduce the risk of intentional sampling (direct impact of hospital, pharmaceutical, human medicine, and municipal wastewaters). All samples were collected in different parts of the mainstream of the river and central points of the lake away from wastewater influence points. Water samples were collected into dark plastic bottles and kept in a cooler with ice until transportation to the laboratory. The sediment samples were collected with spatula to dark plastic jars at the same place where the water samples were collected. Fish samples including common bream (*Abramis brama*), roach (*Rutilus*), pike (*Esox lucius*), zander (*Sander lucioperca*), and catfish (*Silurus*)) were collected at the same place as the water and sediment samples, and kept in a cooler with ice until transportation to the laboratory. In the laboratory, water and sediment samples were stored at -18°C until the analysis. Muscles and skin from each fish sample were prepared, homogenised, and stored at -18°C until the analysis.

Instrumentation. The liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was performed using the Agilent 1200 HPLC system (Agilent Technologies, Germany) with an automatic degasser, a binary pump, and an autosampler connected to the AB Sciex API 4000 triple quadrupole mass spectrometer (AB Sciex, Canada). The chromatographic separation was performed on the Luna C18 (2) 100A column (50 × 4.6 mm, particle size 3 μm, Phenomenex, USA), which was maintained at 30°C. The flow rate of the mobile phase was 400 μL/min and the injection volume was 30 μL.

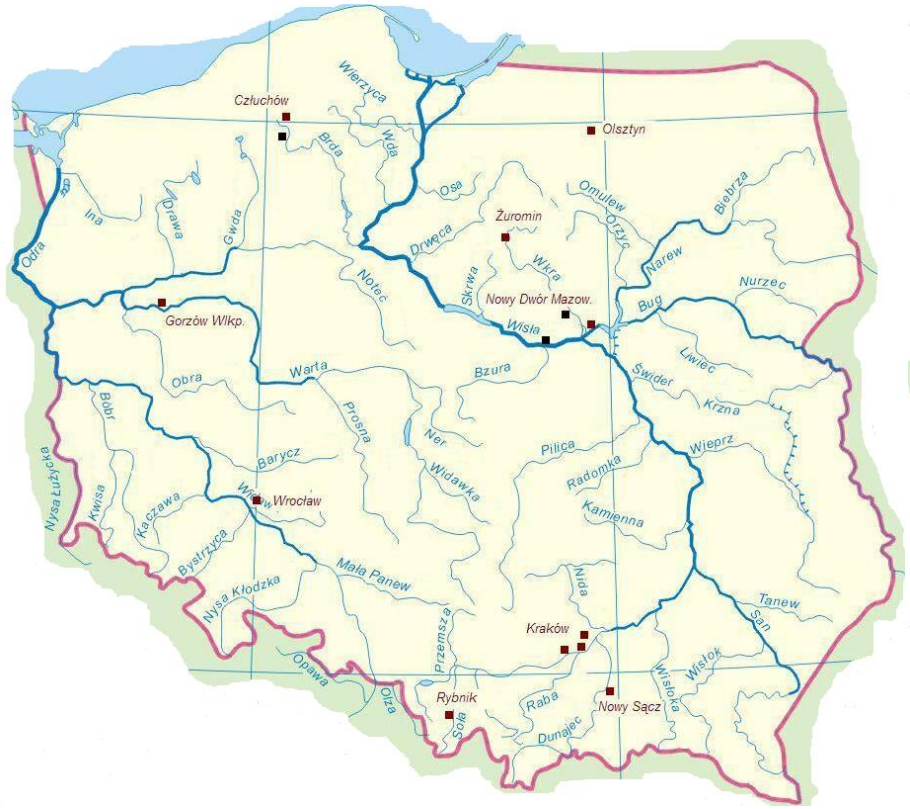


Fig. 1. Locations of sample collection points

Mobile phases A and B were composed of acetonitrile (A) and 0.025% HFBA (B), which was optimized. 85% B started the mobile phase gradient programme, which progressed through 60% B at 1 min, 40% B at 3 min, and finished at 5% at 4 min, held for 3 min. The column returned to the initial composition and it was re-equilibrated for another 6 min before the next injection. The MS instrument was operated in a positive ESI mode. For tuning the following parameters were used: resolutions Q1 and Q3 - unit; temperature - 500°C, nebuliser gas (N_2) - 40; curtain gas (N_2) - 20; collision gas (N_2) - 3; auxiliary gas - 50; ion spray voltage - 5500 V. Analyst 1.5 software spectrometer (AB Sciex, Canada) controlled the LC-MS/MS system and processed the data which was acquired in multiple reaction monitoring (MRM) mode. The ion transitions and mass parameters monitored for all analytes and matrices are listed in Table 1.

Sample preparation.

Fresh water. 50 µL of IS was added to the 250 mL water sample in a 500 mL polypropylene bottle, the solution was mixed, and it was left to incubate at room temperature in a dark place for 15 min. 6 mL of 0.5 M sodium acetate, pH 5.6, and 30 µL of HFBA were added and shaken briefly for 5 min. Strata-X SPE cartridges were conditioned sequentially with 5 mL of methanol, 5 mL of water, and 5 mL of 0.05 M HFBA. Subsequently, the water samples were loaded into the SPE cartridges with 50 mL reservoir at a flow no faster than 1 drop/5 s. Then the cartridges were vacuum-dried for 5 min at a pressure ranging from 12 mmHg to 18 mmHg. The analytes were eluted twice adding 3 mL of acetonitrile and 0.05 M HFBA mixture (9:1, v/v). The eluates were collected in 10 mL glass tubes and evaporated to dryness under a stream of nitrogen at 45 ± 5°C. Finally, the residues were dissolved in 500 µL of 0.025% HFBA and filtered through 0.22 µm PVDF syringe filters into LC vials.

Sediments. One hundred microlitres of IS solution was added to 2 ± 00.1 g of sediment sample before the extraction and the samples were mixed and left to incubate at 4°C in a dark place for 30 min. After adding 6 mL of acetonitrile, 0.5 mL of citric acid, pH 4.0, and 100 µL of 1 M sodium acetate, pH 5.6, the samples were homogenised with a vortex mixer for 2 min. Then the samples were placed in an ultrasonic bath for 15 min, centrifuged at 4500 × g at 5°C for 10 min, and the supernatants were loaded into an Oasis HLB cartridges which was without any preconditioning, the cartridges serving as a filter. The filtered supernatants were collected in glass tubes and evaporated to dryness under a stream of nitrogen at 45 ± 5°C. Finally, the residues were dissolved in 500 µL of 0.025% HFBA and filtered through 0.22 µm PVDF syringe filters into LC vials.

Table 1. Analytes analysed in water, sediments, and fish and their LC-MS/MS parameters

Analyte class	Analyte	Matrices			Ion transition 1 (m/z)	Ion transition 2 (m/z)	DP (V)	CE (Ev)
		water	sediment	fish				
Aminoglycosides	SPEC	+	-	+	351.1/333.2	351.1/207.2	32	67
	STRP	+	-	+	582.0/263.0	582.0/246.0	52	166
	DISTRP	+	-	+	584.3/263.2	584.3/246.2	42	150
	NEO	+	-	-	615.3/161.0	615.3/163.2	109	42
β-lactams	AMOX	+	-	+	366.1/349.1	366.1 /208.0	14	45
	PEN G	+	+	+	335.1/160.0	335.1 /176.1	17	60
	AMPI	+	-	-	350.1/106.0	350.1/160.0	58	27
	DICLOX	+	-	-	470.0/160.0	470.0/311.0	50	22
	NAF	+	-	-	415.0/199.0	415.0/171.0	48	20
	OXA	+	-	-	402.0/160.0	402.0/243.0	52	25
	CFPI	+	+	+	424.0/152.0	424.0/124.0	35	50
	CFT	+	+	+	524.0/241.0	524.0/125.0	25	50
	CFQ	+	+	+	529.0/134.0	529.0/125.0	25	50
	CFLO	+	+	+	459.0/337.1	459.0/152.0	16	46
	CFZ	+	+	+	455.0/323.0	455.0/156.0	15	50
	CFLE	+	+	+	348.0/158.0	348.0/106.0	10	50
	CFPE	+	+	+	646.0/530.0	646.0/530.0	17	60
Diaminopyrimidines	TMP	+	+	+	292.1/262.2	292.1/231.3	52	36
Fluoroquinolones	CIP	+	+	+	332.0/314.0	332.0/231.0	28	65
	ENR	+	+	+	360.0/342.0	360.0/286.0	33	100
	DIF	+	+	+	400.5/382.1	400.5/356.0	30	50
	DAN	+	+	+	358.0/340.0	358.0/255.0	60	33
	FLU	+	+	+	262.1/244.0	262.1/202.0	44	25
	OXO	+	+	+	262.0/244.0	262.0/216.0	53	25
	NAL	+	+	+	233.0/215.0	233.0/187.0	42	30
	MAR	+	+	+	363.0/345.0	363.0/320.0	70	30
	SAR	+	+	+	385.8/368.1	385.8/348.0	50	31
	NOR	+	+	+	320.0/302.0	320.0/231.0	30	50
	ERY	+	+	+	734.0/576.5	734.0/158.2	28	75
Macrolides	TYL	+	+	+	916.0/174.0	916.0/772.5	52	110
	TIL	+	-	-	869.6/696.5	869.6/174.2	135	61
	JOS	+	-	-	828.2/173.9	828.2/229.0	80	46
	SPI	+	-	-	843.5/540.4	843.5/174.2	120	44
Lincosamides	LIN	+	-	-	407.2/126.1	407.2/359.3	74	28
Pleuromutilins	TIM	+	-	-	494.4/192.2	494.4/118.8	128	30
Sulphonamides	SMT	+	+	+	279.2/156.0	279.2/108.0	25	50
	SME	+	+	+	265.0/156.0	265.0/108.0	27	50
	SDMX	+	+	+	311.0/156.0	311.0/108.0	23	50
	SMA	+	+	+	254.0/107.8	254.0/155.9	24	40
	SMM	+	+	+	281.0/156.0	281.0/108.0	35	50
	SFT	+	+	+	256.0/156.0	256.0/108.0	53	20
Tetracyclines	DC	+	+	+	445.0/428.0	445.0/154.0	23	50
	OTC	+	+	+	461.0/426.0	461.0/444.0	28	40
	TC	+	+	+	445.0/410.0	445.0/427.0	27	55
	CTC	+	+	+	479.0/444.0	479.0/462.0	29	60

"+" - analysed analyte, "-"- not analysed analyte

Fish. A hundred microlitres of IS solution was added to 2 ± 00.1 g of fish sample (muscle and skin) before the extraction and the samples were mixed and left to incubate at 4°C in a dark place for 30 min. 0.5 mL of 0.1 M EDTA, 6 mL of 3% meta-phosphoric acid, pH 5.5, 2 mL of 0.02 M HFBA, and 0.6 mL of 20% TCA were added, the samples were homogenised with a vortex mixer for 2 min and mechanically shaken for 10 min, and then centrifuged at 4500 × g at 5°C for 10 min. Then aliquots of supernatants were loaded into Strata X-CW cartridges preconditioned with 3 mL of methanol, water, and 0.02 M HFBA. The cartridges were dried under vacuum for 6 min and eluted twice with 2% formic acid in methanol (3 mL). Following this, the eluates were evaporated to dryness under a stream of nitrogen at 45 ± 5°C. The remaining fish pellets were re-extracted with 6 mL of acetonitrile, vortexed, mechanically shaken for 10 min, and centrifuged at 4500 rpm for 10 min at 5°C. Next, the supernatants were evaporated to dryness under a stream of nitrogen at 45 ± 5°C. Both residues were dissolved in 0.025% HFBA (250 µL) and combined. Finally, the

coupled extracts were filtered through 0.22 µm PVDF syringe filters into LC vials.

Validation procedure. All used analytical methods were validated according to the Commission Decision 2002/657/EC (3) and proved to be sensitive. For the method validation, repeatability, within-laboratory reproducibility, and percentage recovery were determined for each matrix. The overall coefficients of variation (CV) of the fortified samples were calculated for repeatability and within-laboratory reproducibility. The limit of detection (LOD) and limit of quantitation (LOQ) were checked for each matrix (fresh water, fish and sediment) from different sources. The linearity and precision were determined by the matrix-matched calibration curve. Repeatability (CV%) of method for determination of antibiotics for all antibiotic classes was in the range of 4.7% to 12.2% for fresh water samples, 4.1% to 11.8% for sediments, and 2.0% to 12.6% for fish. Within-laboratory reproducibility (CV%) for all antibiotic classes was in the range of 6.8% to 14.4% for fresh water samples, 7.9% to 14.1% for sediment, and 3.8% to 15.0% for fish. The LOQ was in the range of 5 µg/kg to 125 µg/kg for fish samples, 0.02 µg/L to 10 µg/L for fresh water, 1 µg/kg to 8 µg/kg for sediments. The LOD was in the range of 1.7 µg/kg to 84 µg/kg for fish samples, 0.01 µg/L to 3.73 µg/L for fresh water, and 0.8 µg/kg to 4.9 µg/kg for sediments. The overall recoveries ranged from 96% to 111% for fish samples, from 84.3% to 109.3 % for fresh water, and from 93% to 113% for sediments, reference to the internal standard.

Result

No antibiotics at concentrations above the LOQs established for used methods were detected in the examined samples. The results are presented in Table 2.

Table 2. Results of analysis of fresh water, sediment, and fish samples from Polish rivers and lakes

Analyte class	Analyte concentration (µg/L or kg)		
	Fresh water	Sediment	Fish
Aminoglicosides	<1-10	-	<80-125
β-lactams	<0.02-10	<5-8	<10-50
Diaminopyrimidines	<0.05	<5	<10
Fluoroquinolones	<0.02	<2-6	<5-30
Lincosamides	<0.02	-	-
Macrolides	<0.05-5	<5-8	<20-59
Pleuromutilins	<0.02	-	-
Sulphonamides	<0.05	<1-5	<10
Tetracyclines	<0.02-0.05	<1-5	<5-20

Discussion

The control of antimicrobial agents in the aquatic environment has gained much attention, as many have been found in water sources, sediments, and soils. Since the antimicrobials have been widely recognised as new emerging pollutants, the interest in this problem and the development of new methods for the antibiotics monitoring in environmental samples has increased. Because the main pathways of antibiotic discharges to the aquatic environment are pharmaceutical manufacturers, residential care facilities, hospitals, and wastewater treatment plants, most of studies were performed in urban agglomerations. Usually, the studies concerned human and veterinary antibiotics in wastewater (2, 4, 5, 10, 12) and in surface water and sediment from rivers and lakes located in the centre of urban areas (1, 4, 6, 8, 9, 13, 15-17). There are a few publications about antibiotics in farmed fish or shrimps (11, 14). Antibiotic occurrence in aquatic environments is reported especially in China (2, 15-17), USA (5), UK (1), Spain (13), Germany (12), and Portugal (10). Most of these publications reported the presence of sulfonamides (sulfamethoxazole, sulfamethazine,), macrolides (erythromycin), diaminopyrimidines (trimethoprim), tetracyclines (tetracycline, oxytetracycline), and fluoroquinolones (norfloxacin, ciprofloxacin). The antibiotics were identified at concentration levels of a few ng/L to µg/L depending on compound, matrices, and sampling point. Some publications described the occurrence of antibiotics in the Warta river (6) and the seawater of the southern Baltic Sea (1). In the first case, antibacterial compounds were identified at concentrations from 0.22 µg/L to 0.39 µg/L (sulfapiridine), from 0.08 µg/L to 0.27 µg/L (trimethoprim), and from 0.3 µg/L to 0.6 µg/L (sulfamethoxazole). The samples were collected in the wastewater treatment plant in Kozieglowy, 1 km away from Poznan. In the second case, only six out of seven investigated samples contained antibacterial compound residues. Sulfadimethoxine was the most detected compound. In this study, 159 fresh water, 150 sediment, and 443 fish samples were collected far away from urban areas. Some sample collection points were located near livestock farms. Particular attention was paid to the Brda river (impacted by a large number of swine farms) and the Wkra river (influenced by a large number of poultry farms). For the first time in this study, veterinary antibiotics were determined in Polish rivers and lakes. In the samples collected from 14 different river or lake sample collection points, the concentrations of antibacterial compounds were below the limits of quantification. Based on the results of the presented study, it can be concluded that the present of veterinary antimicrobials in Polish river and lake environments is so far not hazardous. There is still not much information about the concentration of human and veterinary antibacterial residues in the Polish

aquatic environment, thus studies like this should be continued in the future.

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