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Occurrence of Fluoroquinolones and Sulfonamides Resistance Genes in Wastewater and Sludge at Different Stages of Wastewater Treatment: A Preliminary Case Study

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Abstract: This study identified differences in the prevalence of antibiotic resistance genes (ARGs) between wastewater treatment plants (WWTPs) processing different proportions of hospital and municipal wastewater as well as various types of industrial wastewater. The influence of treated effluents discharged from WWTPs on the receiving water bodies (rivers) was examined. Genomic DNA was isolated from environmental samples (river water, wastewater and sewage sludge). The presence of genes encoding resistance to sulfonamides (*sul*1, *sul*2) and fluoroquinolones (*qep*A, *aac(6')-Ib-cr*) was determined by standard polymerase chain reaction (PCR). The effect of the sampling season (summer – June, fall – November) was analyzed. Treated wastewater and sewage sludge were significant reservoirs of antibiotic resistance and contained all of the examined ARGs. All wastewater samples contained *sul*1 and *aac(6')-Ib-cr* genes, while the *qep*A and *sul*2 genes occurred less frequently. These observations suggest that the prevalence of ARGs is determined by the type of processed wastewater. The Warmia and Mazury WWTP was characterized by higher levels of the *sul*2 gene, which could be attributed to the fact that this WWTP processes agricultural sewage containing animal waste. However, hospital wastewater appears to be the main source of the *sul*1 gene. The results of this study indicate that WWTPs are significant sources of ARGs, contributing to the spread of antibiotic resistance in rivers receiving processed wastewater.

Keywords: antibiotic resistance; wastewater; WWTP; ARGs; sulfonamides; fluoroquinolones

1. Introduction

The overuse and misuse of antibiotics in human and veterinary medicine, animal farming and agriculture contributes to antibiotic pollution and the spread of antibiotic resistance in the environment [1,2]. The wide use of antimicrobial drugs creates selection pressure, which speeds up microbial evolution and promotes the development of antibiotic resistance mechanisms. The presence of close associations between antimicrobial medicines and resistance has been widely documented around the world [3]. Antibiotics, antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) are ubiquitous in surface water [4–6], ground water [7–9] and soil [10–12]. The ongoing spread of these micropollutants in the natural environment contributes to the development of antibiotic

resistance mechanisms in environmental bacteria. Antibiotic resistance genes play a fundamental role in this process.

The sources and transmission mechanisms of ARGs have to be investigated in detail to prevent the spread of antibiotic resistance in the environment. Raw municipal and hospital wastewater, treated effluents and sewage sludge are significant reservoirs of ARGs, and they play a significant role in the transfer of antibiotic resistance [1,13,14]. For this reason, the release of ARGs from wastewater treatment plants (WWTPs) and their fate in the environment have attracted considerable research interest in recent years [15–19].

The removal of solids, organics and nutrients is the key process in wastewater treatment. Wastewater treatment plants are not equipped to remove microbiological contaminants, including ARB and ARGs. The activated sludge process is a biological method that is frequently used in wastewater treatment. The conditions inside activated sludge tanks, including high oxygen concentration, high temperature and high densities of bacterial cells, can promote horizontal gene transfer (HGT) between closely related as well as unrelated microorganisms [20]. As a result, treated effluents can contain more copies of a given resistance gene per unit volume than raw wastewater [21,22].

Treated wastewater is evacuated to water bodies, which leads to the release of large gene pools, including ARGs, into the natural environment [18,23]. Receptacles of treated effluents accumulate mobile genetic elements (MGEs) such as plasmids, transposons, insertion sequences and integrons [15,24], which are effective carriers of ARGs. Mobile genetic elements play a key role in the spread of genetic information between even the most phylogenetically distant species of bacteria. Genetic resistance can be further transferred between bacteria, including pathogenic microorganisms, posing a significant threat to human health and life.

Organic solid waste such as sewage sludge is also a considerable source of ARGs, which contribute to the spread of antibiotic resistance in the environment. Sewage sludge treatment leads to the recovery of municipal biosolids. Biosolids are abundant in nutrients, and they are often used as agricultural fertilizers [25]. In Poland, the storage of sewage sludge with a high caloric content was banned in 2016 [26], which increased the supply of sewage sludge for fertilization purposes. The management of sewage sludge delivers unquestioned benefits, but the application of treated sludge in agriculture may also have negative consequences. Research has shown that ARGs levels in treated sewage are very high [27,28] and often significantly exceed ARGs concentrations in wastewater [29,30]. Sewage sludge is usually stabilized before it is used as fertilizer, but according to many authors, biosolids processed with the use of various stabilization methods are still highly abundant in ARGs [30,31]. Elevated concentrations of ARGs in soil samples [28,30,32] and accelerated transmission of ARGs from soil to plants [32] were also reported in farmland fertilized with sewage sludge.

Microorganisms have developed various mechanisms of resistance to antibiotics, depending on the type of antimicrobial drugs and their effects on bacterial cells. In Europe, clinical bacterial isolates are becoming increasingly resistant to fluoroquinolones, which are an important class of antibiotics [33]. Resistance to fluoroquinolones is encoded by *aac(6′)-Ib-cr* and *qep*A genes [34], which are usually localized in plasmid DNA. The *qep*A gene encodes efflux pumps of the major facilitator superfamily (MFS), which are responsible for the transport of antibiotics to extracellular space. The *aac(6′)-Ib-cr* gene encodes an enzyme that suppresses the activity of two fluoroquinolone antimicrobials (ciprofloxacin and norfloxacin) through their acetylation [35].

Sulfonamides are one of the oldest groups of antibiotics used in medicine. In recent years, the use of sulfonamides in human medicine has declined due to growing levels of bacterial resistance [36], but these antimicrobials are still widely applied in veterinary medicine [37]. Sulfonamides inhibit folate synthesis by suppressing the production of dihydropteroate synthase (DHPS) (EC 2.5.1.15), an enzyme involved in the synthesis of folic acid. Bacteria have developed mechanisms of resistance against sulfonamides through mutation of the chromosomal DHPS gene (*folP*) or the acquisition of an alternative DHPS gene (*sul*). Dihydropteroate synthase encoded by a *sul* gene has a low affinity for sulfonamides, and is not inhibited by this class of antibiotics. Three genes encoding resistance

to sulfonamides (*sul*1, *sul*2, *sul*3) with estimated 50% sequence similarity have been identified to date [37,38]. It is believed that general resistance to sulfonamides is encoded mostly by *sul*1 and *sul*2. The acquisition of sulfonamide resistance through *sul* genes is the most prevalent mechanism in the environment [19,39].

This study aimed to determine the prevalence of genes encoding resistance to sulfonamides (*sul*1, *sul*2) and fluoroquinolones (*qep*A, *aac(6')-Ib-cr*) in various stages of wastewater treatment in two WWTPs. The examined plants are situated in the regions of Warmia and Mazury (northern Poland) and Silesia (southern Poland), which differ considerably in industrial development level. The treatment plants use similar methods of wastewater treatment based on activated sludge. Differences in the prevalence of ARGs between the studied WWTPs processing different proportions of the hospital and municipal wastewater as well as various types of industrial wastewater were investigated. The study analyzed whether the inflow of various sources of industrial wastewater (brewery wastewater, animal industry wastewater) affects the differentiation in the occurrence of ARGs in WWTPs. Samples were collected at the subsequent stages of wastewater treatment to capture the ARGs reduction, and the influence of treated effluents evacuated from WWTPs on the receiving water bodies was examined by analyzing samples of river water collected upstream and downstream from wastewater discharge points. Sewage sludge was also analyzed. The impact of the season on the ARGs prevalence was evaluated by analyzing samples collected in summer (June) and fall (November).

2. Materials and Methods

2.1. Study Area and Sampling Sites

In this study, the presence of genes encoding resistance to fluoroquinolones and sulfonamides was analyzed in two WWTPs with similar treatment systems and processing capacities. Two wastewater treatment plants (WWTPs) in Poland were analyzed in the study. The WWTPs are located in different Polish regions: Warmia and Mazury (WM-WWTP) and Silesia (S-WWTP). Treated effluents are evacuated to the Łyna River and the Gostynia River, respectively. Samples of river water collected upstream and downstream from effluent discharge points were examined to determine the influence of wastewater processing technology on rivers receiving treated wastewater. The analyzed WWTPs are characterized by similar treatment technologies, but they differ in the type of inflowing wastewater. Both WWTPs have a similar daily average processing capacity and employ a mechanical-biological treatment system based on the activated sludge. S-WWTP has an average influent flow rate of 32,000 m^3/day, and it receives municipal sewage from the city of Tychy, hospital wastewater and industrial sewage. Industrial wastewater (25% of the receiving wastewater) is supplied mainly by a brewery where sewage is pre-cleaned in the methane fermentation process. The brewery is the largest and the most important industrial facility in the S-WWMT area. S-WWTP deploys mechanical-biological treatment methods and operates sequencing C-TECH reactors, activated sludge chambers (a system of chambers of various oxygen conditions: anaerobic, anoxic and aerobic), a secondary settling tank and an anoxic chamber. S-WWTP uses supplementary chemical phosphorus removal [40]. WM-WW TP operates a mechanical-biological treatment system with an elevated removal of nutrients (MB-ERN) with the following sections of the wastewater treatment process: a pre-denitrification chamber, a phosphorus removal tank, nitrification/denitrification chambers and secondary settling tanks. It processes municipal sewage from the city of Olsztyn, industrial wastewater and sewage from three hospitals. Industrial wastewater (20% of the receiving wastewater) is supplied by animal industry wastewater. According to the information obtained from the administration of the WM-WWTP, the plant has an average processing capacity of 35,000 m^3/day. The prevalence of bacterial infections, the amount of antimicrobial drugs and the number of hospitalized patients differ across seasons. These parameters can influence the results noted in each sampling season. Therefore, samples were collected in the summer (June) and fall (November) of 2018. Samples of river water, wastewater and

sludge from various stages of treatment were analyzed (n = 36). The types of samples collected in each WWTP are presented in Table 1.

Table 1. Types of collected samples.

	Region of Silesia (S-WWTP)* (n = 18)		Region of Warmia and Mazury (WM-WWTP)** (n = 18)	
	Type of Sample	**Symbol**	**Type of Sample**	**Symbol**
Liquid samples from WWTPs	Untreated wastewater	S1	Untreated wastewater	W1
	Wastewater from the outlet of the primary clarifier	S2	Wastewater from the outlet of the primary clarifier	W2
	Wastewater from the outlet of the secondary clarifier	S3	Wastewater in the biological chamber	W3
	Wastewater from the outlet of the C-TECH reactor	S4	Wastewater from the outlet of the multipurpose reactor	W4
	Treated wastewater	S5	Treated wastewater	W5
Solid samples from WWTPs	Sludge from the outlet of the mechanical concentrator	S6	Sludge from the outlet of the open fermentation pool	W6
	Sludge from the outlet of the gravity concentrator	S7	Treated sludge	W7
River water	River water upstream the effluent discharge point	S8	River water upstream the effluent discharge point	W8
	River water downstream the effluent discharge point	S9	River water downstream the effluent discharge point	W9

* Silesian wastewater treatment plant; ** Warmia and Mazury wastewater treatment plant.

Three grab samples of around 160 mL of wastewater or river water were individually collected and combined into composite samples in sterile 500 mL bottles. Sludge samples were collected into sterile urine containers. The samples were transported to the laboratory on the day of collection and stored in 4 °C for further analysis.

2.2. DNA Extraction

Samples of wastewater and river water were filtered with the use of vacuum pumps and passed through 0.2 µm pore size polycarbonate membrane filters. The volume of the filtered samples ranged from 10 mL to 400 mL, depending on the site and season of collection. Sludge samples of 0.25 g each were used directly for DNA isolation.

The DNeasy Power Water Kit (Qiagen, Hilden, Germany) was used to isolate genomic DNA from sewage and water samples, and the DNeasy Power Soil Kit (Qiagen, Hilden, Germany) was used to isolate genomic DNA from sewage sludge samples according to the manufacturer's protocol. The quality and quantity of the obtained genetic material was checked with the Multiskan Sky Microplate Spectrophotometer (Thermo Scientific, Waltham, MA, USA).

2.3. Identification of Aantibiotic Resistance Genes by Polymerase Chain Reaction (PCR)

The presence of genes encoding resistance to sulfonamide (*sul*1, *sul*2) and quinolone (*qep*A, *aac (6')-Ib-cr*) was confirmed by PCR. The applied primers and reaction profiles are presented in Table 2. The PCR reactions were performed in a volume of 15 µL containing 10 µM of the respective primer pairs, 1 µL of genomic DNA of each sample and the NZYTaq II 2xGreen Master Mix.

PCR products were separated electrophoretically by transferring 5 µL of every amplified DNA fragment to 1.5% agarose gel stained with ethidium bromide (0.5 µg/mL) (Sigma, St. Louis, MO, USA). Electrophoresis was conducted for 1 h at 100 V in 0.5× TBE buffer.

Table 2. Polymerase chain reaction (PCR) primers and parameters.

Target Gene	Primer Sequence (5′-3′)	Amplicon Size (bp)	PCR Annealing Temp (°C)	References
*sul*1	CGCACCGGAAACATCGCTGCAC	163	55.9	[41]
	TGAAGTTCCGCCGCAAGGCTCG			
*sul*2	TCCGGTGGAGGCCGGTATCTGG	191	60.8	
	CGGGAATGCCATCTGCCTTGAG			
*qep*A	CCAGCTCGGCAACTTGATAC	570	58	[42]
	ATGCTCGCCTTCCAGAAAA			
aac(6′)-Ib-cr	TTGCGATGCTCTATGAGTGGCTA	482	55	[43]
	CTCGAATGCCTGGCGTGTTT			

2.4. Cluster Analysis

An analysis of hierarchical clustering was performed to illustrate the relationship of the sampling sites and the collection season based on the occurrence of studied ARGs. The Dice similarity coefficient (Sørensen–Dice index) was used to measure the relationship between two sets of data. The unweighted pair group method with arithmetic mean (UPGMA) was used as a clustering method. A hierarchical tree (dendrogram) of the analyzed samples was generated for each research object (Supplementary materials). The clustering analysis was performed using the Molecular Evolutionary Genetics Analysis (MEGA7, Pennsylvania State University, State College, PA, USA, 2016) software [44].

3. Results and Discussion

The results of the cluster analysis did not show a similarity between the results for samples collected in the same season. The samples collected in different seasons from the same sampling sites were often grouped in one cluster on the dendrogram. It can be concluded that the sampling season probably had no effect on the presence of ARGs in the analyzed samples (Figure S1). However, the prevalence of ARGs differed across the types of genes and the stages of wastewater treatment. Raw sewage was a significant source of ARGs and contained all of the analyzed genes (Table 3).

Influent wastewater is an important reservoir of ARGs. Wastewater treatment plants offer supportive conditions for the spread of ARGs by horizontal gene transfer (HGT) [45–47]. The low effectiveness of treatment processes can contribute to the transmission of ARGs from treated effluents to surface water [2,19,48]. Antibiotic resistance genes are localized on MGEs, which can be exchanged by both closely related and phylogenetically distant bacterial species. As a result, antibiotic resistance can be spread between bacteria of anthropogenic origin and bacterial communities in the natural environment [6,42]. ARGs in the environment stays in two forms: intracellular ARGs (iARGs) and extracellular ARGs (eARGs). The forms of ARGs that occur in the environment determine the character of its further transmission. iARGs support the spreading of antibiotic resistance via conjugation and transduction, while eARGs can be uptaken and integrated by the competent non-resistant bacteria via natural transformation. Of the mentioned HGT mechanisms, conjugation is deemed to have the biggest impact on the dissemination of ARGs, while transformation and transduction are considered less important [49].

Sulfonamides were the first safe and effective antimicrobial drugs in clinical practice that targeted selected bacteria [50]. The medical use of sulfonamides is decreasing in Europe. The compound annual growth rate (CAGR) of the sulfonamide market in Europe reached negative values between 2009 and 2018 [36]. In 2017, sulfonamides were the least used class of antibiotics for systemic use in Polish and European hospitals [51]. Sulfonamide resistance in Gram-negative bacteria can probably be attributed to *sul*1 and *sul*2 genes, which are carried by plasmids. Research has demonstrated that sulfonamide resistance genes are the most ubiquitous ARGs in the environment [19,39]. In the

present study, the *sul*1 gene was identified in all wastewater and sewage sludge samples collected in both WWTPs. This gene was also present in samples of river water collected upstream from the effluent discharge point. The above findings indicate that the *sul*1 gene is widely spread in the environment. The analyzed gene was not eliminated in successive stages of treatment, which may increase the concentration of this gene in the water and bottom sediments of effluent-receiving rivers. Surface waters are highly contaminated with the *sul*1 gene [15,16,18,52]. The *sul*1 gene has been also identified in samples of municipal wastewater [53], activated sludge [47] and wastewater containing animal waste [54]. Hospital sewage appears to be the major source of the *sul*1 gene in the environment. According to Lye et al. [2], the prevalence of *sul*1 is considerably higher in hospital wastewater than in other types of sewage. Similar results were reported by Wang et al. [14], who analyzed hospital sewage in China. In other studies, hospital wastewater was characterized by the highest concentration of the *sul*1 gene relative to other ARGs [13,55]. The ubiquitous character of *sul*1 could result from a close relationship between *sul*1 and class 1 integrons that are widespread in the environment [56]. According to Poey et al. [57], *sul*1 occurs in the variable region (gene cassette) of class 1 integrons. In the current study, hospital sewage accounted for 2–6% of the wastewater processed by the WWTPs. WM-WWTP treats wastewater from three hospitals, and S-WWTP treats wastewater from one hospital. Hospital sewage could be a major source of *sul*1 in the analyzed WWTPs, which could explain the high prevalence of this gene in all samples.

Table 3. Presence of amplicons for genes encoding resistance to sulfonamides and fluoroquinolones in the analyzed samples. Colors correspond to sampling sites as in Table 1. + and − denote amplicons for each gene that were and were not detected via endpoint PCR, respectively.

	Symbol	*sul*1		*sul*2		*qepA*		*aac(6′)-Ib-cr*	
		Summer	Fall	Summer	Fall	Summer	Fall	Summer	Fall
S-WWTP *	S1	+	+	+	+	+	+	+	+
	S2	+	+	+	+	−	+	+	+
	S3	+	+	+	−	−	+	+	+
	S4	+	+	+	−	−	−	+	+
	S5	+	+	+	−	−	+	+	+
	S6	+	+	−	+	−	−	+	+
	S7	+	+	+	+	+	+	+	+
	S8	+	+	−	+	−	−	+	+
	S9	+	+	−	+	−	−	+	+
WM-WWTP **	W1	+	+	+	+	+	+	+	+
	W2	+	+	+	+	+	+	+	+
	W3	+	+	+	+	−	+	+	+
	W4	+	+	+	+	+	−	+	+
	W5	+	+	+	−	+	+	+	+
	W6	+	+	+	+	−	−	+	+
	W7	+	+	+	+	+	−	+	+
	W8	+	+	−	−	−	−	+	+
	W9	+	+	+	−	−	+	+	+

* Silesian wastewater treatment plant; ** Warmia and Mazury wastewater treatment plant.

The *sul*2 gene occurred less frequently than the *sul*1 gene. The *sul*2 gene was not identified in river water upstream from the effluent discharge point (excluding site S8 in fall). This gene was present in all samples of raw wastewater, but it was eliminated in successive stages of treatment. These observations could also suggest that *sul*2 was less abundant in raw sewage that *sul*1. The presence of the *sul*2 gene in river water was noted only in WM-WWTP in summer. Koczura et al. [52] reported significantly smaller concentrations of *sul*2 than *sul*1 in surface waters. In a study by Ziembińska-Buczyńska et al. [47], *sul*2 was less prevalent than *sul*1 in bacterial isolates from activated sludge. According to Pei et al. [41], *sul*2 is an important indicator of the environmental impacts of agriculture, which could explain why *sul*2 concentrations are higher in areas with a predominance of agriculture, in particular livestock

production and aquaculture. Lye et al. [2] found the highest abundance of *sul*2 in zoo wastewater. The livestock population in Warmia and Mazury is several times higher than in Silesia [58], which could imply that Warmia and Mazury is characterized by a higher contamination of animal waste and higher concentrations of *sul*2 in the environment, in particular in wastewater evacuated from animal farms. Olsztyn, the capital city of Warmia and Mazury, where MW-WWTP is situated, is the seat of Poland's largest poultry company, which specializes in turkey rearing and the production and processing of turkey meat. Wastewater from turkey farms and production plants is evacuated to MW-WWTP, which could explain the higher abundance of *sul*2 in raw sewage reaching MW-WWTP than S-WWTP. The *sul*2 gene was not identified in only one stage of wastewater treatment in MW-WWTP, whereas in S-WWTP, *sul*2 was not detected in four sampling sites.

According to the literature, seasonal variations in the virulence of pathogenic microorganisms and the immune status of infected individuals are responsible for the seasonality of infectious diseases [59]. Seasonal variations are also noted in antibiotic use, and the consumption of antimicrobials is significantly higher in fall than in summer [60]. Despite the above, it appears that the prevalence of sulfonamide resistance genes in samples of river water, wastewater and sludge was not noticeably affected by season in the present study; however, this is only a preliminary case study and to determine more precise correlations the research must be expanded with quantitative analyses of ARGs. Similar observations were made by Koczura et al. [52], who did not report significant seasonal differences in *sul*1 copy numbers in river water and sewage sludge, but noted that the *sul*2 gene was more prevalent in spring samples.

Fluoroquinolones are broad-spectrum antibiotics and one of the most frequently prescribed antimicrobials. Extensive clinical use of fluoroquinolones has contributed to high resistance of pathogenic microorganisms to this group of antibiotics. In Europe, the number of hospital strains of *Escherchia coli* and *Klebsiella pneumoniae* resistant to fluoroquinolones increased in 2015 –2018. In 2018, the highest percentage of *Pseudomonas aeruginosa* isolates (19.7%) were resistant to fluoroquinolones [33]. The genes conditioning bacterial resistance to fluoroquinolones include *aac(6′)-Ib-cr*, which encodes aminoglycoside acetyltransferase, and *qep*A, which encodes active efflux pumps [34]. In this study, both genes were detected in samples of raw wastewater.

The *aac(6′)-lb-cr* gene was identified in all analyzed samples, and it was not eliminated during wastewater treatment. It was also detected in samples of river water collected upstream from effluent discharge points, which suggests that this gene also originates from other sources. The *qep*A gene was less frequently isolated, and it was not found in river water sampled upstream from effluent discharge points. In S-WWTP, the *qep*A gene was completely eliminated from treated wastewater in summer, and it was not transmitted to the river with the evacuated effluents. This gene was present only in sewage sludge where the concentration of biological material was highest relative to the remaining samples. The *qep*A gene was less effectively removed in WM-WWTP. In both seasons, *qep*A was not detected in river water sampled upstream from the effluent discharge point, but it was identified in the samples collected downstream from the effluent discharge point.

According to the literature, *aac(6′)-lb-cr* and *qepA* genes are commonly found in the wastewater. Korzeniewska and Harnisz [21] detected both genes in influents and effluents from 13 wastewater treatment plants with different type of the wastewater treatment technologies modifications. Yan et al. [61] found *aac(6′)-lb-cr* and *qep*A in treated wastewater from three Chinese hospitals, in municipal wastewater and in river water. In each sampling site, the *aac(6′)-lb-cr* gene had higher copy numbers than the *qep*A gene. Wen et al. [48] analyzed samples of unpolluted river water and samples of river water collected in the vicinity of hospitals. The *qep*A gene was not detected in any of the samples, whereas the *aac(6′)-lb-cr* gene was ubiquitous in all samples.

The lower prevalence of *qep*A could be attributed to the fact that resistance to fluoroquinolones encoded by this gene evolved relatively late. The *qep*A gene was first identified in 2007 by research teams from Belgium and Japan [62,63]. The first mechanism of plasmid encoded resistance to fluoroquinolones was discovered in 1998 [64].

It should be noted that all studied genes (excluding *qep*A at site W7 in fall) were present in treated sewage sludge intended for further use. More than 500,000 tons of sewage sludge are produced each year in Poland, of which around 20% are used as agricultural fertilizers on account of their high organic matter content [65]. The application of sewage sludge as agricultural fertilizer contributes to the spread of antibiotic resistance in the environment [28]. Chen et al. [28] found that sewage sludge fertilization can transfer up to 108 of ARGs and MGEs to soil. The resulting increase in bacterial diversity in the soil environment was significantly correlated with the prevalence of ARGs. Sewage sludge is particularly abundant in tetracycline and sulfonamide resistance genes [27]. According to Lee et al. [27], sulfonamide resistance genes (*sul*1 and *sul*2) were the most frequently occurring ARGs (45.6%) in the examined sewage sludge. The number of *sul*1 copies in sewage sludge significantly exceeded (26–87 times) the number of *sul*2 copies. Lee et al. [27] also observed that the prevalence of sulfonamide resistance genes was six-fold (%) higher in sewage sludge than in raw wastewater, which suggests that these genes are highly accumulated in sewage sludge. Quinolone resistance genes were identified significantly less frequently, and they were not present in sewage sludge. The accumulation of ARGs and MGEs in soil could imply that the application of sewage sludge could accelerate gene transmission to the soil environment via HGT.

The presence of antibiotics acting as active mutagens can also significantly contribute to the evolution of drug resistance mechanisms. Sublethal concentrations of antibiotics, which are frequently noted in wastewater, can lead to mutations that promote the emergence of drug resistance [66,67]. The samples of raw sewage, treated wastewater and river water that were examined in the current study were additionally analyzed by Giebułtowicz et al. [68] for the presence of 26 antimicrobials. Very high concentrations of sulfamethoxazole (SXT) and ciprofloxacin (CIP) were noted in raw wastewater reaching both WWTPs, and they were nearly three times higher in WM-WWTP than in S-WWTP. It should also be noted that CIP levels in raw wastewater exceeded the minimum inhibitory concentrations (MIC) recommended by EUCAST for most microorganisms [69]. To regulate the risk issuing from the concentrations of antibiotics in the environment, Bengtson-Palme and Larsson [70] estimated Predicted No-Effect Concentrations (PENCs) of antimicrobials for resistance selection. Based on these [70] calculations, average CIP concentrations in influents, effluents and downstream rivers were found to exceed the PENCs values for influents, effluents and downstream rivers in both WWTPs, which is particularly worrying. Based on the calculated values of the risk quotient (RQ), Giebułtowicz et al. [68] concluded that high concentrations of the analyzed antibiotics contribute to the development of selective resistance. The results of the qualitative analyses presented in this study cannot reveal a correlation between antibiotic concentrations and the prevalence of the examined ARGs. Further research involving quantitative analysis is needed to investigate the above problem.

The study determined whether the tested ARGs are present/absent in the DNA isolated from analyzed samples. However, it remains unknown if and what part of the current ARGs is intercellularly carried (iARGs) by live and metabolically active bacterial cells. Therefore, to reach beyond the qualitative and quantitative analyzes of ARGs and to reach a more extensive knowledge of ARGs dynamics, it is necessary to identify which of the detected ARGs are expressed. The combination of metagenomic and metatranscriptomic analyses will allow the identification of ARGs that are not only present but also actively transcribed. There is little work on antibiotic resistance gene expression in WWTP [71–73]. The researchers report that about 65.8% identified ARGs shows transcriptional activity [73], and that there is a significant overexpression of ARGs occurring in an environment that is heavily affected by antibiotic use [71]. We believe that this issue should be the direction of further research in the WWTPs.

4. Conclusions

Wastewater treated at WWTPs is a significant point source of ARGs. The existing wastewater treatment methods do not effectively eliminate ARGs whose concentrations in treated effluents are not routinely monitored. This imperfect process promotes the release of ARGs into the environment.

This study demonstrated that genes encoding resistance to sulfonamides and fluoroquinolones are widespread in the environment and that WWTPs contribute to their transmission. The prevalence of ARGs in raw wastewater and in various stages of wastewater treatment differed in the examined WWTPs. These variations could be attributed to differences in the type and sources of processed wastewater. WM-WWTP processes wastewater from livestock farms, which could explain the higher prevalence of the *sul*2 gene in the analyzed samples. Hospital wastewater appears to be the main source of *sul*1 in both WWTPs. Sewage sludge was found to be a significant reservoir of ARGs. Sewage sludge should be stabilized before it is used as fertilizer, and its application in agriculture should be monitored. The growing prevalence and spread of ARB and ARGs pose a significant public health concern around the world. A sound knowledge of the sources and transmission mechanisms of antibiotic resistance in various environments is required to develop effective strategies for managing these risks and evaluating their impact on human health. The study is a preliminary study and a base for further in-depth metagenomic and metatranscriptomic analyses.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3417/10/17/5816/s1, Figure S1: Similarity of samples, based on the occurrence of antibiotic-resistance genes (ARGs).

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