



Impact of Hospital Wastewater on the Occurrence and Diversity of Beta-Lactamase Genes During Wastewater Treatment with an Emphasis on Carbapenemase Genes: A Metagenomic Approach

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The diversity of beta-lactam antibiotic resistance genes, with particular emphasis on carbapenemase genes, during the treatment process at two wastewater treatment plants (WWTPs) with different levels of hospital wastewater inflow was investigated using high-throughput sequencing. An additional aspect of the study was to determine the taxonomic diversity of microorganisms in the studied samples. The obtained results suggest that bacteria of the Fusobacteriaceae family, not associated to date with this phenomenon, may be involved in the spread of antibiotic resistance in the environment. In samples from both wastewater treatment plants, the dominant beta-lactamase genes included blaOXA, blaGES, blaBEL, blaCfxA, and blaTEM. It is worth noting that the blaKPC and blaNDM genes were only found in untreated municipal wastewater with a higher hospital wastewater content. Moreover, an increase in the abundance of the blaIMP gene after the biological treatment stage in the studied treatment plants was found. In wastewater characterized by a higher proportion of hospital wastewater, 94 correlations were observed, while in wastewater with its lower proportion, 41 correlations were noted. Considering the above, the current research indicates that the inflow of hospital wastewater contributes to the spread of antibiotic resistance in the aquatic environment.

Keywords: wastewater, hospital wastewater, antibiotic resistance genes, environmental resistance, beta-lactamases, carbapenemases

INTRODUCTION

Antibiotics belonging to the beta-lactam group are used in human medicine in the greatest quantities (Fernandez et al., 2017). In its 2018 report, the World Health Organization (WHO) stated that beta-lactam antibiotics are the most commonly used antibiotics worldwide (WHO, 2018). The WHO Collaborating Centre classifies beta-lactam antibiotics into two groups: 1) beta-lactam antibacterials, 2) penicillins and other beta-lactam antibacterials, including carbapenems. Antibiotic consumption

is determined using the Defined Daily Dose (DDD) unit per 1,000 inhabitants per day. In a 2020 report, the European Centre for Disease Prevention and Control (ECDC) determined the average antibacterial consumption to be 19.4 DDD per 1,000 inhabitants per day in Europe. The European country with the highest consumption of antibiotics from the above-mentioned groups was Greece. The Netherlands had the lowest consumption, with Poland ranked 11th among European countries (ECDC, 2020; Buta et al., 2021).

Given the very high use of beta-lactam antibiotics, genes conferring resistance to this group of antibiotics have begun to pose a hazard to public health due to their widespread occurrence. Beta-lactam resistance genes can be classified and divided into four classes (the Ambler classification) (Bush, 2018). Classes A, C, and D include beta-lactamases with a serine active center, while class B includes enzymes with auxiliary divalent zinc cofactor (Bonomo, 2017). Penicillinase, first described in 1940, belonged to class C and is now known as AmpC present in *Escherichia coli* (Abraham and Chain, 1940). The literature mostly mentions the beta-lactamases originating from microorganisms possessing AmpC, including MIR, DHA, ACC, MOX, EBC, and FOX types, with the CMY-2 subtype being the most widespread one of them (Nikonorow et al., 2013; Chaturvedi et al., 2020). Many of the class A and D beta-lactamases are classified as extended-spectrum beta-lactamases (ESBLs) such as TEM, SHV, and CTX-M which are among the most abundant ESBLs worldwide. Other, less commonly found ESBLs belonging to the GES, PER, and VEB types, have also been described (Dallenne et al., 2010). Ghafourian et al. (2015) reported that over 100 TEM-type beta-lactamases were determined, of which only TEM-1 and TEM-2 did not encode ESBLs. Moreover, the presence of a wide range of SHV and CTX-M beta-lactamase variants was confirmed. The above-mentioned beta-lactamases (TEM, SHV, and CTX-M) have been identified in many representatives of the *Enterobacteriaceae* family (*Escherichia coli*, *Klebsiella pneumoniae*), and in bacteria of the *Pseudomonas* and *Acinetobacter* genera. It is noteworthy that while the above-mentioned microorganisms are associated with the natural environment (river water) or wastewater, they can also cause infections in hospitals. Of all the beta-lactamase classes, carbapenem antibiotic-hydrolyzing beta-lactamases (very important to public health) are currently found in three of them, i.e., classes A, B, and D. The most commonly discussed class A carbapenemase-encoding genes include KPC and GES types; for class B—NDM, IMP, and VIM types; for class D, these include OXA-23, -24, -51, -58, and OXA-48-related subtypes. Frequently described variants of carbapenemase-encoding gene types in municipal wastewater, hospital wastewater and river water include NDM-1, NDM-5, NDM-7, KPC-2, VIM-1, VIM-2, GES-5, and OXA-48 (Yang et al., 2016; Nasri et al., 2017; Mathys et al., 2019).

A particular hazard to human health and life is posed by Gram-negative bacteria carrying beta-lactamase genes, which are capable of rapidly acquiring subsequent antibiotic resistance determinants (Khurana et al., 2017). Currently, the greatest threat to hospital patients' health is presented by ESBL-encoding genes conferring resistance to penicillins as well as

first-, second-, and third-generation cephalosporins and aztreonam (Sonda et al., 2016), and genes encoding carbapenemases hydrolyzing carbapenem antibiotics. Both enzyme groups are produced by bacteria of the *Enterobacteriaceae* family and other Gram-negative bacteria (Khan et al., 2017). The hospital outbreaks triggered by the above-mentioned microorganisms are bacterial infections which are very difficult to treat (Müller et al., 2016). The presence of ESBL-producing bacteria was found on various usable hospital surfaces (Muzslay et al., 2017). Rapid dissemination of these antimicrobial-resistant bacteria, coupled with the unavailability of effective antibiotics, contributed to the warning issued by the WHO of the "post-antimicrobial era" in which the world will be afflicted by widespread infections (Nwafia et al., 2019; Matar et al., 2020).

In recent years, numerous studies have demonstrated the presence of antibiotic-resistant bacteria (ARB), antibiotic resistance genes (ARGs), and unmetabolized antibiotics in hospital or municipal wastewater, as well as the possible release of these pollutants into the natural environment (Röderová et al., 2016). In hospital wastewater, both ESBL-encoding genes (*bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M}) (Adelowo et al., 2018; Haller et al., 2018; Banjo et al., 2020) and carbapenemase-encoding genes (*bla*_{KPC}, *bla*_{VIM}, *bla*_{IMP}, *bla*_{OXA-48}, *bla*_{NDM}) (Nasri et al., 2017; Jin et al., 2018; Cahill et al., 2019) were detected. The literature indicates that hospital wastewater, as compared to municipal wastewater, features a significantly higher diversity and abundance of ARGs, including ESBL-, and carbapenemase-encoding genes (Hassoun-Kheir et al., 2020).

Since wastewater treatment plants employing conventional treatment methods do not completely reduce antibiotic-resistant bacteria and drug resistance genes found in the inflowing wastewater (Osińska et al., 2020; Zieliński et al., 2020), more knowledge is needed on the fate of ARGs originating from hospital wastewater during the treatment process. It is also necessary to determine the role played by hospital wastewater inflowing to WWTP in the spread of antibiotic resistance genes in the environment. Therefore, this study aimed to determine the diversity of beta-lactam resistance genes, with particular emphasis on carbapenemase genes, during the treatment process at two WWTPs receiving different levels of hospital wastewater inflow. The metagenomic analysis employed in the study enabled the determination of the taxonomic diversity of microorganisms in the studied samples.

MATERIALS AND METHODS

Sampling Sites

Samples for analysis were collected from two WWTPs with different levels of hospital wastewater inflow relative to the total amount of wastewater reaching the treatment facilities. The first WWTP is located in the region of Warmia and Mazury (WM-WWTP), and the second WWTP is located in the region of Silesia (S-WWTP) in Poland (Europe).

Although both WWTPs deploy mechanical and biological wastewater treatment systems, additional phosphorus chemical removal technology was implemented in S-WWTP. WM-WWTP processes wastewater from the capital city of the region of Warmia and Mazury (Northern Poland) and four surrounding municipalities and it has an average daily processing capacity of 35,000 m³. The plant receives wastewater from four hospitals, which accounts for 2% of the total treated wastewater. WM-WWTP has a population equivalent (PE) of 260,361, and treated wastewater is characterized by a chemical oxygen demand (COD) of 49 mg/L and biological oxygen demand (BOD) of 6 mg/L. The processing technology involves mechanical treatment systems with automated screens for separating large solids, two horizontal sand filters and two sedimentation tanks. The biological treatment system is composed of phosphate and nitrogen removal chambers and five multifunctional bioreactors equipped with four Passavant aerating rotors each and secondary sedimentation tanks. Sewage sludge is inactivated in closed and open digestion chambers and it is dehydrated by a mechanical filter press. Processed wastewater is discharged to a river (Kowalska et al., 2014).

S-WWTP (Southern Poland) receives 32,731 m³ of wastewater each day and has a PE of 403,138. Hospital wastewater accounts for 0.4% of the wastewater processed by S-WWTP. Wastewater entering the plant via trunk collectors is pumped to a pre-treatment line composed of hook screens, belt conveyors, and a manual screen for the separation of large solids. Pretreated wastewater is then passed through horizontal sand filters. Filtered wastewater undergoes biological treatment in the C-TECH reactor. In the first stage, phosphorus is precipitated chemically with an iron coagulant. Wastewater is then directed to the reactor where it is treated in a repeated series of aeration, sedimentation, and decantation processes. The last stage of biological treatment involves phosphorus removal, denitrification, and nitrification. The generated sewage sludge is neutralized in anaerobic digestion chambers and it is dehydrated in a high-speed decanter centrifuge and a belt filter press (RCGW, 2020). Schematic diagrams of the analyzed WWTPs are presented in **Supplementary Figures S1, S2** in the supplementary materials.

Sample Collection

Samples of wastewater and sewage sludge were collected in June (2018) (summer), November (2018) (autumn), and March (2019) (spring). Wastewater and sewage sludge were sampled from different stages of treatment in both WWTPs. The sampling sites are described in detail in **Supplementary Table S1; Supplementary Figures S1, S2**.

Samples of wastewater were collected into sterile 500 ml bottles (SIMAX) and sewage sludge samples were collected using 100 ml sterile plastic tubes. Wastewater was sampled in triplicate at hourly intervals for 24 h to obtain representative samples and the collected hourly samples were pooled into a composite sample. Samples of sewage sludge were also collected in triplicate to create complex samples. The samples were transported to the laboratory at a temperature of 4°C.

DNA Extraction and Sequencing

Metagenomic DNA was extracted from wastewater using the Power Water (MoBio Laboratories Inc., CA, United States) kit and from sewage sludge using the Power Soil kit (MoBio Laboratories Inc., CA, United States) following the manufacturer's instructions. Samples were quantified by the picogreen method using Victor 3 fluorometry and their quality and quantity were assessed. Illumina TruSeq DNA PCR-Free libraries were prepared manually following the manufacturer's protocol TruSeq DNA PCR-Free Sample Preparation Guide, Part #15036187 Rev. D (Illumina, San Diego, CA, United States). The libraries were tested using LightCycle qPCR and the size distribution was assessed by an Agilent Technologies 2100 Bioanalyzer using a DNA 1000 chip and sequenced on a NovaSeq6000 in an S4 flowcell lane using a 2 × 150 bp configuration.

Bioinformatic Analysis

Forty-five samples from three seasons were used for metagenomics analysis. Fastq sequences were initially quality filtered, including adaptor-trimming using Trimmomatic v 0.39 (Bolger et al., 2014) with default parameters. Paired-end sequences were merged using PANDAseq (Masella et al., 2012). Reads that software could not merge were excluded from further analyses. The data sequences then underwent quality control using the fastx toolkit 0.0.14-5 (http://hannonlab.cshl.edu/fastx_toolkit/) with the fastq_quality_filter tool. Data filtration excluded all reads from analyses that did not reach a minimum of 80% of bases set at a quality score of q = 20. Using Seqtk (<https://github.com/lh3/seqtk>), fastq files were converted to fasta files for the BLAST alignment.

Subsequently, metagenomic sequences were analyzed by a BLASTx- type search against the Comprehensive Antibiotic Resistance Database (CARD) (McArthur et al., 2013) (<https://card.mcmaster.ca>; October 2019 3.0.7). Samples were parsed and filtered to collect the top hits for each sample considering positive-hit reads with an *E* value ≤ 1 × 10⁻¹⁰ (Escudeiro et al., 2019); amino acid identity ≥90% (Casaburi et al., 2019) and alignment length ≥25 amino acids (Kristiansson et al., 2011; Zhang et al., 2011; Yang et al., 2013; Su et al., 2017).

Database of integrons and insertion sequences was created by downloading DNA sequences from the INTEGRALL (<https://integrall.bio.ua.pt>). Sequences belonging to integrons or IS-like sequences were identified by aligning the sequences against sequences in the database using BLASTn with *E* value ≤ 1 × 10⁻¹⁰ (Tian et al., 2016). A read was annotated to be a resistance gene if its BLASTn hit for the alignment against the INTEGRALL database had a ≥90% nucleotide read identity for ≥100 bp.

Sequencing results in the form of FASTQ files were uploaded to the MetaGenome Rapid Annotation Subsystems Technology (MG-RAST) server for analysis (6). Each file underwent quality control (QC), which included quality filtering (removing sequences with ≥5 ambiguous base pairs), and length filtering (removing sequences with a length ≥2 standard deviations from the mean). The UCLUST algorithm was used to cluster the identified rRNA sequences. A representative sequence of each cluster was used for taxonomic identification based on the

Greengenes reference database. The metagenome data are deposited in the NCBI Sequence Read Archive (SRA) under accessions NCBI SRA SRP286056.

For further analyses, the results obtained from samples collected from three research seasons (summer, autumn, and spring) were averaged.

Statistical Analysis

To investigate the co-occurrence patterns of microbial community and resistome, correlation matrixes were constructed by calculating all pairwise Spearman rank correlations. A correlation between any two items was considered statistically robust if the Spearman correlation coefficient (ρ) was >0.7 and the p value was <0.01 (Barberán et al., 2012). The resulting correlation matrixes were translated into an association network using Gephi 0.9.1 (Bastian et al., 2009).

RESULTS

Overview of Metagenomic Analysis

The study used Illumina deep sequencing to identify ARGs, mobile genetic elements (MGEs), and the taxonomic structure in samples of wastewater and sewage sludge. The sequencing enabled generating >250 Gb of raw data from 15 samples and a number of reads ranging from 94.8 to 129.5 million (Supplementary Table S2). The databases identified the occurrence of 1,906 ARGs and class 1, 2, and 3 integron integrase genes. Antibiotic resistance genes from the beta-lactamase group accounted for 67.3% of genes in samples from WM-WWTP and for 62.7% of genes from S-WWTP. The study focused mainly on carbapenem resistance genes, i.e., the bla_{GES} , bla_{IMP} , bla_{KPC} , bla_{NDM} , bla_{VIM} gene types and bla_{OXA-23} , bla_{OXA-24} , bla_{OXA-48} , and bla_{OXA-58} gene subtypes. Only subtypes conferring resistance to carbapenems (GES-11, -14, -18, -2, -20, -4, -5, -6) were selected from the bla_{GES} type gene group (Naas et al., 2016; Bonomo, 2017).

Taxonomic Structure

In the studied samples, 22 phyla from the *Bacteria* domain (82%) and three phyla from the *Archaea* domain (12%) were identified. Moreover, the occurrence of 17 classes from four phyla (*Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Euryarchaeota*) was determined and attention was paid to three families because of the association of these families with beta-lactamases and frequent causation of nosocomial infections (*Enterobacteriaceae*, *Moraxellaceae*, and *Fusobacteriaceae*) (Supplementary Figures S2–S4). The data concerning microorganism diversity, averaged for three seasons (including standard deviations) at particular sampling sites, are presented in Supplementary materials (Supplementary Tables S3–S8).

Statistical analysis demonstrated an absence of statistically significant differences between the occurrence of specific microorganism phyla, classes, or families in wastewater at subsequent stages of the treatment system ($p > 0.05$) (Supplementary Tables S8–S10). Of all the phyla found in

wastewater from both wastewater treatment plants, *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* (38.5 and 39.6%, 31 and 29%, 9.1 and 14.9%, respectively in WM-WWTP and S-WWTP) were dominant (Supplementary Figure S3). At each stage of wastewater treatment, in both wastewater treatment plants, the microorganisms belonging to the *Proteobacteria* phylum were most abundant. A change in the proportion of these microorganisms between the inflowing and treated wastewater (52.5%:51%, and 48.4%:35.6%, percentage distribution in WM1:WM5 and S1:S5, respectively) was noted (Supplementary Figure S3). Moreover, a reduction in the number of the most abundant phyla of microorganisms during the wastewater treatment process amounted to 75.5%/87.8% for *Proteobacteria*, 58.1%/57.6% for *Bacteroidetes*, and 91.5%/94.1% for *Firmicutes*, in WM-WWTP/S-WWTP, respectively. The results indicate a reduction in the count of bacteria from the *Firmicutes* phylum in wastewater from both treatment plants, following the process of treatment using activated sludge (WM4/S3) when comparing WM1/S1 to WM4/S3 (23.6%/26.6%:3.4%/4.3%). In addition, the C-TECH reactor used in the S-WWTP decreased the count of these microorganisms with lower efficiency, when comparing the percentage occurrence of the *Firmicutes* phylum in wastewater from reactor S4 (14.8%) to the activated sludge chamber S3 (4.3%) (Supplementary Figure S3).

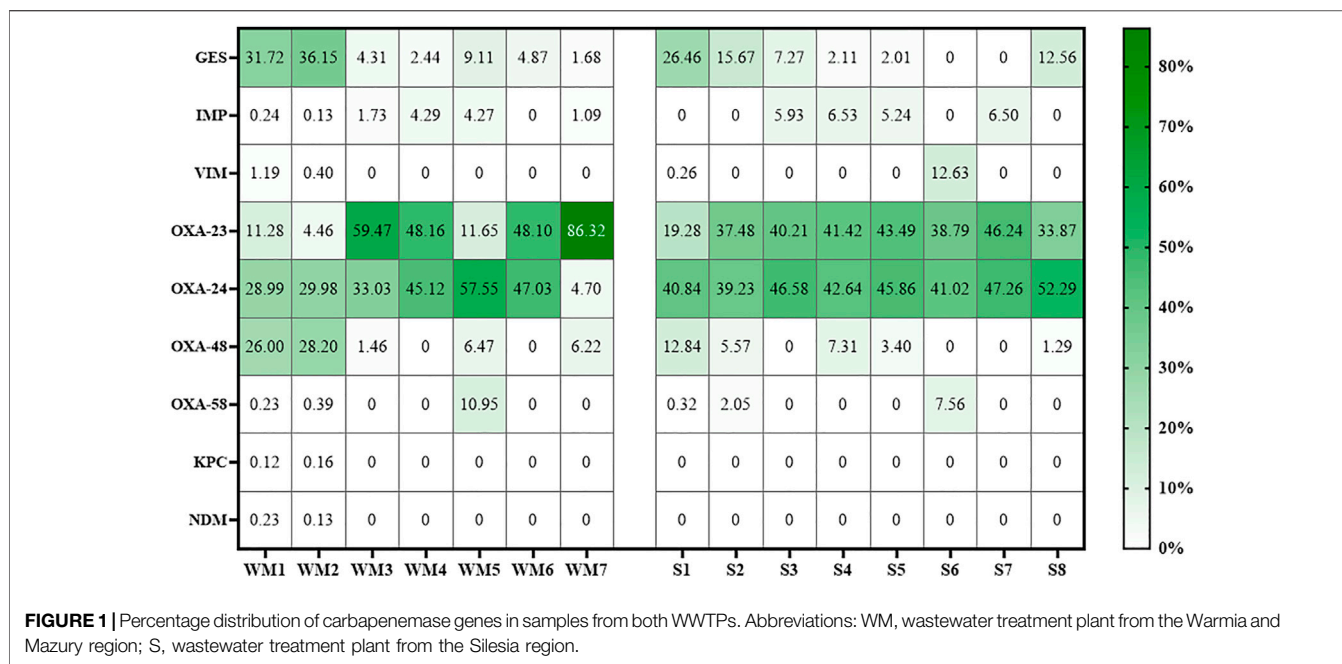
For the *Euryarchaeota* phylum (Archaea), the authors observed an increase in these microorganism counts up to 12% in the sample of sewage sludge (WM6) and leachate from sewage sludge (S6) for both studied wastewater treatment plants (Supplementary Figure S1). A deeper taxonomic analysis of this phylum determined the dominant proportion of the *Methanomicrobia* class in samples from these sites, amounting to 17 and 12%, respectively (Supplementary Figure S4).

Selected microorganism families (*Enterobacteriaceae*, *Moraxellaceae*, and *Fusobacteriaceae*) occurred more abundantly in samples from WM-WWTP than in the samples from S-WWTP and the *Moraxellaceae* family had the highest count (in wastewater from both treatment plants). In the samples of inflowing wastewater and treated wastewater from both treatment plants, the reduction in the count of microorganisms belonging to the mentioned families amounted to 85–99%.

Abundance of ARGs and MEs

In the studied samples, the presence of beta-lactam antibiotic resistance genes was detected, i.e. 1,283 genes encoding beta-lactamases were assigned to 58 types in samples from WM-WWTP and 1,195 genes represented 52 types in samples from S-WWTP (Supplementary Figures S6, S7). The particular numbers of gene copies are expressed in the “ppm” unit which represents “gene copy per 1 million reads.”

In samples from both wastewater treatment plants, the dominant beta-lactamase gene types included bla_{OXA} , bla_{GES} , bla_{BEL} , bla_{CfXA} , and bla_{TEM} . In samples from the WM-WWTP, these genes occurred in the following ranges: 0.064–1.415 ppm, 0.008–2.358 ppm, 0.014–0.388 ppm, 0.008–0.98 ppm, and 0.008–0.758 ppm, while in samples from S-WWTP, these genes occurred in the following ranges: 0.041–1.765 ppm,



0.008–1.224 ppm, 0.019–0.425 ppm, 0.01–1.458 ppm, and 0.02–0.559 ppm, respectively (Supplementary Tables S12, S13). The difference in the number of gene copies determined in wastewater samples between the experimental treatment plants was not statistically significant ($p > 0.05$) (Supplementary Table S14). In samples from both treatment plants, the most abundant carbapenem resistance genes included the *bla*_{GES} type and the *bla*_{OXA-23}, *bla*_{OXA-24}, and *bla*_{OXA-48} subtypes (Figure 1).

No statistically significant differences were found in the abundance of the studied beta-lactam resistance genes between raw wastewater (WM1/S1) and treated wastewater (WM5/S5) ($p > 0.05$) (Supplementary Table S15). However, during the treatment process, a percentage increase was observed in the number of gene types occurring in the highest amounts in wastewater (*bla*_{OXA}, *bla*_{BEL}, *bla*_{CfxA}, and *bla*_{TEM}) (Supplementary Figures S6, S7).

Even though both studied wastewater treatment plants are based on the activated sludge technology, differences were found in the occurrence of certain beta-lactamases in wastewater samples from activated sludge chambers. Based on the percentage occurrence, an increase in the number of *bla* genes in samples after the treatment stage using activated sludge (WM4/S3) was noted for samples from a site with the inflowing wastewater (WM1/S1) (*bla*_{OXA} 2.7-fold, *bla*_{BEL} 4.5-fold in WM-WWTP, and *bla*_{TEM} 2.9-fold, *bla*_{BEL} 5.7-fold in S-WWTP) (Supplementary Figures S6, S7). It is worth noting the difference in the number of genes in samples from S-WWTP after the treatment site in the activated sludge chamber (S3) and after the C-TECH reactor (S4). The use of the C-TECH reactor reduced the number of genes in the effluent (Supplementary Figures S6, S7).

The processes employed at the initial treatment stage (WM1/WM2/S1/S2) enabled a great reduction in the abundance of

carbapenemase genes from the inflowing wastewater. The *bla*_{NDM} and *bla*_{KPC} genes, which were only present in the wastewater inflowing to WM-WWTP, were eliminated from the wastewater after the mechanical treatment stage. For the *bla*_{IMP} gene, in both treatment plants, the biological treatment system increased the amount of this gene (0.24:4.27% in wastewater from WM-WWTP, and 0.00:5.24% in wastewater from S-WWTP, WM1:WM5, and S1:S5, respectively) (Figure 1).

The occurrence of three mobile genetic elements, i.e., *intI1*, *intI2*, and *intI3*, was determined in the studied samples. Among these elements, in each studied sample from both treatment plants, *intI1* constituted the majority (>98%) (Supplementary Figure S8). In samples from the WM-WWTP, integrase genes occurred in the following ranges: 328.59–2911.29 ppm, 0.36–34.4 ppm, and 2.41–10.02 ppm, while in samples from S-WWTP, they were detected in the following ranges: 166.59–2883.91 ppm, 0.02–29.87 ppm, and 0.09–2.46 ppm, for *intI1*, *intI2*, and *intI3*, respectively (Supplementary Tables S16, S17).

Correlation Analysis of ARGs, MGEs and Taxonomic Structure

Spearman’s rank statistical test demonstrated a higher number of statistically significant correlations ($p < 0.01$) between integrase genes and beta-lactam resistance genes in samples from WM-WWTP than in samples from S-WWTP (Figures 2, 3). In wastewater from the treatment plant receiving a higher percentage of hospital wastewater in the total volume of wastewater under treatment (the Warmia and Mazury region), statistically significant correlations were determined between the abundance of antibiotic resistance genes and *intI* genes, amounting to 38 for *intI1*; 33 for *intI2* and 23 for *intI3*, while

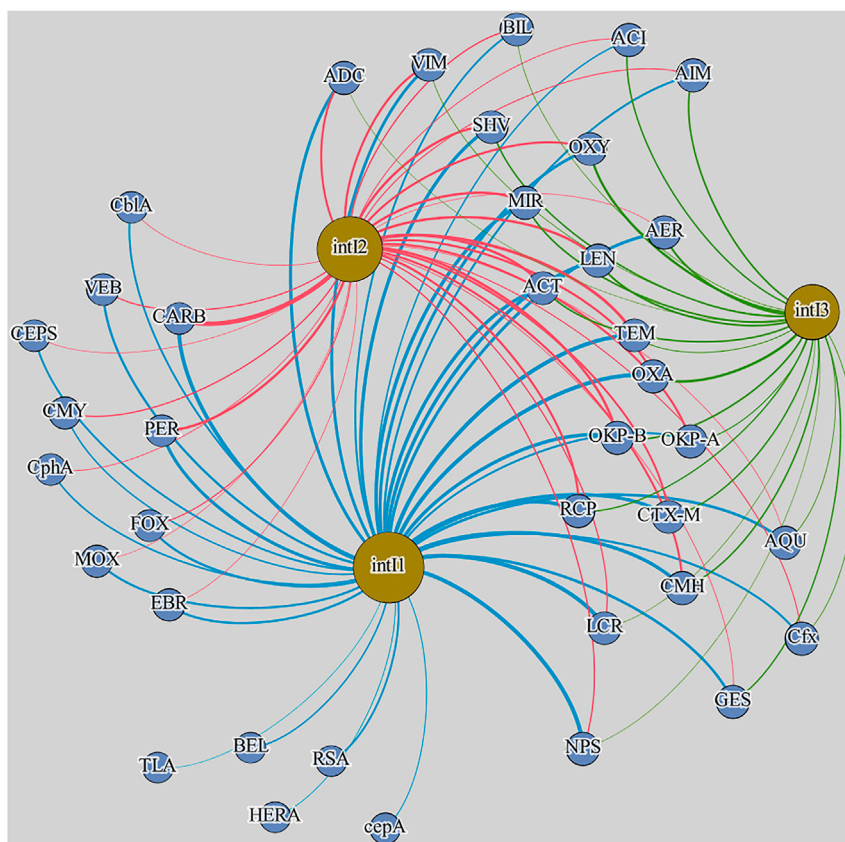


FIGURE 2 | Network analysis of integrase and beta-lactamase genes in samples from WM-WWTP. All correlations in the diagram are statistically significant ($p < 0.01$). Edges: blue—*int1*:beta-lactamase gene correlations, yellow—*int2*:beta-lactamase gene correlations, pink—*int3*:beta-lactamase gene correlations; Nodes: yellow—integron integrase genes, blue—beta-lactamase genes. WM-WWTP stands for the wastewater treatment plant from the Warmia and Mazury region.

in the treatment plant with a lower percentage of hospital wastewater (the Silesia region)—it amounted to 22, 16, and 3 for *int1*, *int2*, and *int3*, respectively. In samples from WM-WWTP, the presence of strong, positive, statistically significant correlations was observed between the abundance of the three studied integrase gene types and carbapenemase genes *bla*_{GES}, *bla*_{VIM}, *bla*_{OXA-24}, *bla*_{OXA-48}, *bla*_{OXA-58}. The respective correlation coefficients were 0.57–0.74, 0.56–0.75, 0.64–0.79, 0.58–0.81, 0.58–0.7. In contrast, in samples from S-WWTP, the abundance of *int1* and *int2* genes correlated less strongly with the genes *bla*_{GES}, *bla*_{OXA-23}, *bla*_{OXA-24}, *bla*_{OXA-48} than in samples from WM-WWTP (0.52–0.59, 0.5–0.64, 0.5–0.62, 0.58–0.62, respectively).

In samples from WM-WWTP, the abundance of the identified bacterial phyla, *Fusobacteria* and *Proteobacteria*, correlated strongly with class 1 integron integrase genes (0.56 and 0.45), class 2 integron integrase genes (0.53 and 0.45), and class 3 integron integrase genes (0.44 and 0.31). However, all of the above-mentioned correlations were statistically insignificant except for the correlation between the count of bacteria from the *Fusobacteria* phylum and the *int1* gene.

In samples from S-WWTP, only the number of class 1 and 2 *int1* genes correlated strongly with the number of microorganism phyla. The abundance of genes *int1* correlated strongly with the abundance of two phyla, *Fusobacteria* (0.59), and *Proteobacteria* (0.58), while the abundance of *int2* correlated strongly with the abundance of up to 10 phyla, i.e., *Firmicutes* (0.79), *Euryarchaeota* (0.72), *Actinobacteria* (0.7), *Tenericutes* (0.63), *Bacteroidetes* (0.61), *Synergistetes* (0.59), *Spirochaetes* (0.55), *Crenarchaeota* (0.54), *Cyanobacteria* (0.52), and *Fusobacteria* (0.5).

Samples from the treatment plant in the Silesia region were characterized by a higher number of statistically significant correlations between the abundance of microorganism phyla and carbapenem resistance genes. In samples from WM-WWTP, this only included the phylum of *Fusobacteria*, while in samples from S-WWTP, these phyla included *Fusobacteria*, *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Dictyoglomi*.

An analysis of Spearman's correlations between the count of selected microorganism families and the abundance of integrase and carbapenemase genes determined a higher number of significant positive correlations between variables in samples from WM-WWTP (22 correlations) compared to samples

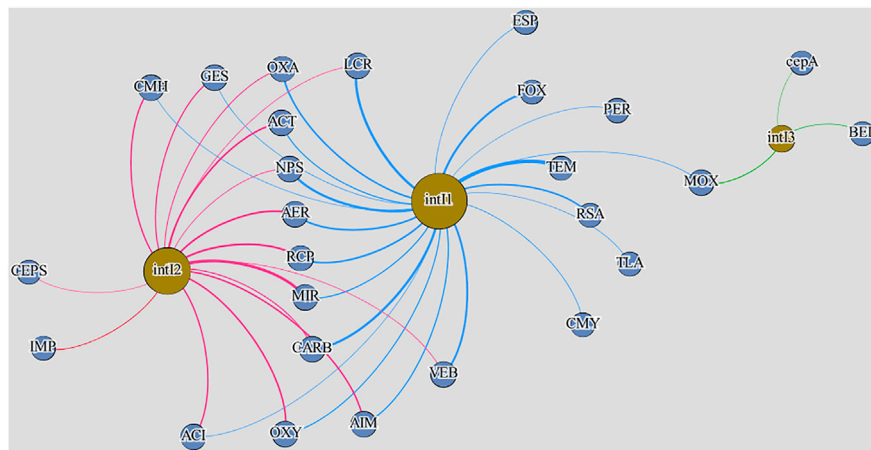


FIGURE 3 | Network analysis of integrase and beta-lactamase genes in samples from S-WWTP. All correlations in the diagram are statistically significant ($p < 0.01$). Edges: blue—intI1:beta-lactamase gene correlations, yellow—intI2:beta-lactamase gene correlations, pink—intI3:beta-lactamase gene correlations; Nodes: yellow—integron integrase genes, blue—beta-lactamase genes. S-WWTP stands for the wastewater treatment plant from the Silesia region.

from S-WWTP (19 correlations). In samples from both treatment plants, the abundance of *intI1* and *intI2* genes correlated significantly with the abundance of the families of *Enterobacteriaceae*, *Moraxellaceae*, and *Fusobacteriaceae*, however, only in samples from WM-WWTP can the emergence of significant correlations between the numbers of *intI3* gene and *Fusobacteriaceae* (0.44) and *Moraxellaceae* (0.51) be observed. In samples from both treatment plants, the abundance of *Fusobacteriaceae* and *Moraxellaceae* correlated strongly with the number of carbapenem resistance genes (Supplementary Figures S9, S10).

DISCUSSION

The taxonomic structure of the studied samples collected from the wastewater treatment plant was typical of samples of this type (Ye et al., 2016; Guo et al., 2017). The study results indicate the dominant proportion of bacteria from the *Proteobacteria*, *Firmicutes*, and *Bacteroidetes* phyla in the taxonomic structure of the studied samples. The literature confirms that bacteria from the *Proteobacteria* phylum are dominant in wastewater samples, even though the species belonging to this phylum account for a small percentage of the human gastrointestinal biota (Bäumlisberger et al., 2015). Microorganisms from the *Firmicutes* phylum, along with *Bacteroidetes*, are also among those most commonly found in wastewater samples (Singh et al., 2012; Wang et al., 2014). In the presented study, bacteria from the *Firmicutes* phylum occurred in higher numbers at the initial stages of the treatment process and their percentage in wastewater decreased over the subsequent stages of the treatment process. Bacteria belonging to the human gastrointestinal biota, including the *Firmicutes* phylum, often shape the taxonomic structure of inflowing wastewater, while at the later stages of treatment, their percentage is effectively reduced (Cai et al., 2014).

For an in-depth taxonomic analysis, the *Enterobacteriaceae* and *Moraxellaceae* families representing the *Proteobacteria* phylum were selected. These families include species carrying carbapenem resistance genes and causing outbreaks of nosocomial infections, such as carbapenem-resistant *Enterobacteriaceae* (CRE), and carbapenem-resistant *Acintebacter baumannii* (CRAB). Currently, these species pose one of the greatest hazards to public health. Infections caused by these organisms are often linked to high morbidity and mortality (Grundmann et al., 2017; Iovleva and Doi, 2017; Logan and Weinstein, 2017; Isler et al., 2018; David et al., 2019; Hamidian and Nigro, 2019). Moreover, the study focused on microorganisms from the *Fusobacteriaceae* family since an analysis of the obtained study results indicated that these microorganisms may be a significant factor in harboring antibiotic resistance genes in the environment.

The study found that a greater diversity of beta-lactam resistance gene types was found in wastewater inflowing to WM-WWTP (which contained a higher percentage of hospital wastewater) than in samples from S-WWTP. The literature confirms that the inflow of hospital wastewater may induce a greater diversity of antibiotic resistance genes, including among beta-lactam antibiotic resistance gene types (Buelow et al., 2018; Khan et al., 2019). Researchers commonly describe hospital wastewater as a source of a wide spectrum of antibiotic-resistant bacteria and antibiotic resistance genes (Chagas et al., 2011; Korzeniewska and Harnisz, 2013; Hocquet et al., 2016; Rowe et al., 2017). The literature also identified a greater amount of drug resistance genes in wastewater originating from hospitals than in exclusively municipal wastewater by comparing the occurrence of beta-lactam antibiotic resistance genes (Zagui et al., 2020). The genes occurring most abundantly in samples from both treatment plants included *bla*_{TEM} and *bla*_{OXA}. Numerous literature reports confirm the presence of these genes in environmental samples (river water, wastewater) at high

concentrations (Amador et al., 2015; Zieliński et al., 2019; Wang et al., 2020; Chaturvedi et al., 2021).

Based on the research results presented in this study, it was found that one of the carbapenem resistance genes, occurring in abundance in wastewater in both studied treatment plants, was *bla*_{GES}. The literature indicates the occurrence of carbapenemase-resistance subtypes of *bla*_{GES} in both wastewater (Conte et al., 2017; Wang et al., 2020) and bacterial strains isolated from wastewater (Kim, 2016; Araújo et al., 2021). As regards another carbapenemase-encoding gene (*bla*_{IMP}), a significant increase in its amount was found in wastewater from both treatment plants after the biological treatment stage, which contributed to an increase in its occurrence in treated wastewater. At the stage of wastewater treatment in the activated sludge chamber, an increase in the number of antibiotic resistance genes can take place, which is most likely due to the high density of bacterial cells and the induced horizontal transfer of genes between microorganisms (Jiao et al., 2017). Jendrzewska (2018) described a case of an increase in the amount of carbapenem resistance genes (*bla*_{GES} and *bla*_{PER}) at the biological treatment stage. Makowska et al. (2020) also indicated an increase in the concentration of genes such as *bla*_{KPC} or *bla*_{NDM} in wastewater after treatment using the biological method. Moreover, Wang et al. (2015) compared various activated sludge-based wastewater treatment systems and noted that an aeration tank with activated sludge induced an increase in the concentration of antibiotic resistance genes, which may pose a threat to the natural environment. Metagenomic analysis studies identified an activated sludge tank as a hotspot of various ARGs, independent of wastewater source, or treatment processes (Liu et al., 2019). Moreover, the literature describes the occurrence of carbapenem resistance genes in river water receiving treated wastewater (Chouchani et al., 2013; Khan et al., 2019). It is noteworthy that the genes *bla*_{NDM} and *bla*_{KPC} were present in the wastewater inflowing to WM-WWTP. Both genes are characteristic of the bacteria strongly associated with outbreaks of nosocomial infections, such as *Klebsiella pneumoniae* (Nasri et al., 2017; Teixeira et al., 2020) or *Acinetobacter baumannii* (Goudarzi et al., 2019). Lamba et al. (2017) concluded that the concentration of carbapenem-resistant bacteria of the *Enterobacteriaceae* family and the *bla*_{NDM} gene was nine orders of magnitude higher in hospital wastewater than in local municipal wastewater.

Beta-lactamase and carbapenemase encoding genes are usually located on plasmids (the structure of which often includes integrons) or in the bacterial chromosome (Papp-Wallace et al., 2016; Perez et al., 2016; Bonomo, 2017). The literature identifies the presence of beta-lactam resistance genes from all Ambler classes in integron gene cassettes in environmental samples such as treated wastewater, sewage sludge, or soil (Gatica et al., 2016; Böhm et al., 2020). Despite sequence differences in the integrase gene, it was described that the same gene cassettes could be found in class 1 and 2 and class 1 and 3 integrons (Carattoli, 2001). In 1995, the occurrence of the same gene cassette carrying the *bla*_{IMP} gene in class 3 integron, which was initially determined in class 1 integron, was described for the first time (Arakawa et al., 1995). In addition, the literature

reports on the possibility of the occurrence of gene cassette rearrangement between class 1 integrons and class 3 integrons in an environment with a rich microbiome and resistome, such as wastewater (Stalder et al., 2012; Amos et al., 2015).

The metagenomic analysis conducted in this study shows the dominant proportion of the class 1 integron integrase gene in samples from both wastewater treatment plants. Mobile genetic elements such as *intI1* genes are effective tools for bacterial adaptation and play a significant role in the transmission of antibiotic resistance. Previous studies have noted that anthropogenic environmental changes lead to the promotion of the abundance of integrase genes, particularly the *intI1* gene (Stalder et al., 2012). The differences in the amounts of integrase genes demonstrated in this study, have also been confirmed in the literature (Guo et al., 2017; Piotrowska et al., 2017; Wang et al., 2017; An et al., 2018). Integrase genes are found in higher concentrations in wastewater of hospital origin (Timraz et al., 2017; Wang et al., 2018) compared to municipal wastewater (Di Cesare et al., 2016), which may explain the occurrence of the studied *intI1* in higher amounts in samples from WM-WWTP.

Based on the analysis of links between the studied variables (beta-lactam resistance genes and genes encoding the integrase enzyme), more significant positive correlations were found in samples from the wastewater treatment plant receiving a higher load of hospital wastewater (WM-WWTP). The obtained study results suggest the increasing importance of the *intI3* gene in antibiotic resistance gene dissemination in samples from wastewater treatment plants that receive hospital wastewater in addition to municipal wastewater. Positive, significant correlations were found between the amount of *intI3* with an abundance of *bla*_{VIM} and *bla*_{GES} genes. In their study, Lamba et al. (2017) also described significant correlations between the abundance of beta-lactamase genes (*bla*_{CTX}, *bla*_{NDM}, *bla*_{TEM}, and *bla*_{OXA}) and the number of class 1 and 3 integrons, while their study results show that the abundance of class 2 integrons did not significantly correlate with the number of resistance genes. Simo Tchuinte et al. (2016) also highlight the role of class 3 integron in the spread of the antibiotic resistance phenomenon while describing the occurrence of this mobile genetic element harboring oxacillinase genes in effluent hospital wastewater. Shibata et al. (2003) found the occurrence of *intI3* gene in clinical strains carrying genes from the metallo-β-lactamase group. Moreover, class 3 integrons may be involved in the spread of resistance both under clinical conditions and in the natural environment and may participate in the exchange of antibiotic resistance genes between these two environments (Barraud et al., 2013).

Wastewater constitutes a rich reservoir of bacteria and genes. The numerous links between them are well described (Yuan et al., 2018; Ju et al., 2019). In this study, the occurrence of the dominant *Proteobacteria* phylum was strongly related to the abundance of integrase- and carbapenemase-encoding genes. However, in the samples from WM-WWTP which received a major volume of hospital wastewater, highly statistically significant correlations were found between the occurrence of the *Fusobacteria* phylum and the abundance of carbapenemase genes, despite the occurrence of these bacteria in small amounts.

Bacteria belonging to this phylum are mostly part of the natural human oral microbiota (McInnes et al., 2020). The literature reports on the occurrence of strong correlations between this phylum and the genes encoding resistance to aminoglycosides, beta-lactams, and antibiotics from the group of macrolides, lincosamides, and group B streptogramins (MLS_B) (Zhou et al., 2018) or genes from the oxacillinase group (Ambler class D) (Qiu et al., 2019). To the best of the authors' knowledge, to date there have been no reports in the available literature on the important role of this phylum in spreading the antibiotic resistance problem in wastewater. This study, however, confirms the role of *Fusobacteria* in the spread of ARGs, including the carbapenemase-encoding genes of importance to public health.

CONCLUSION

The employed metagenomic analysis determined the hospital wastewater inflowing to WWTP as a probable factor leading not only to an increase in the number of carbapenemase-encoding genes but also to an extension of the diversity of resistance gene types in wastewater. In wastewater with a higher percentage of hospital wastewater, as compared to wastewater with its lower percentage, *bla*_{KPC} and *bla*_{NDM} genes, associated with dangerous hospital outbreaks, were detected. Wastewater with a higher load of hospital wastewater was characterized by a greater number of statistically significant correlations between an abundance of specific beta-lactamase gene types and an abundance of integrase gene types compared to wastewater with a lower percentage of hospital wastewater. Microorganisms belonging to the families of *Moraxellaceae* (such genera as *Acinetobacter* sp. and *Moraxella* sp.) and *Fusobacteriaceae* (which are strongly linked to the described mobile genetic elements and carbapenem resistance genes) may be a significant factor in the spread of resistance genes. The obtained results provide new information on the possible involvement of bacteria of the *Fusobacteriaceae* family in the spread of antibiotic resistance in the environment. It is also worth highlighting the increasing importance of *intl3* gene interactions between antibiotic resistance genes and bacteria in wastewater with a higher percentage of hospital wastewater.

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Considering the above, the results of this study show the widespread occurrence of antibiotic resistance genes (including very clinically important carbapenem resistance genes) in municipal wastewater with hospital wastewater inflow. Moreover, this paper describes how the studied wastewater may contribute to the spread of those pollutants in the aquatic environment irrespective of the treatment system.

DATA AVAILABILITY STATEMENT

The metagenome data used in submitted paper #738158, are deposited in the NCBI Sequence Read Archive (SRA) under accessions NCBI SRA BioProject: PRJNA666519—<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA6665196>.

AUTHOR CONTRIBUTIONS

Conceptualization, SC, MH, EK, GP; Methodology, SC, MH, EK, GP; Soft-ware, JH, TD; Validation, JH; Formal Analysis, JH, SC; Data Curation, JH, SC, TD, ŁJ; Writing—Original Draft Preparation, JH; Writing—Review and Editing, SC, MH, EK, GP; Visualization, JH; Supervision, MH; Funding Acquisition, MH, GP.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fenvs.2021.738158/full#supplementary-material>

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