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Environmental fate of *Bacteroidetes,* with particular emphasis on *Bacteroides fragilis* group bacteria and their specific antibiotic resistance genes, in activated sludge wastewater treatment plants

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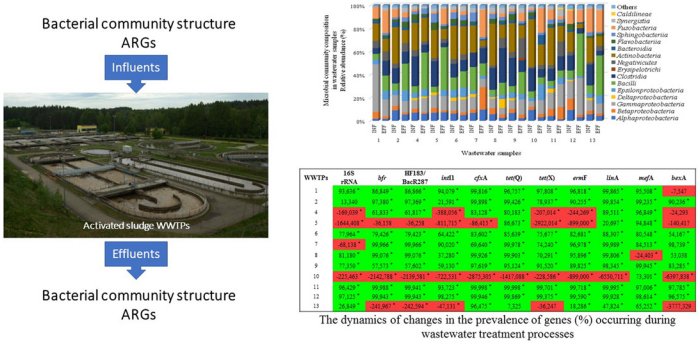
GRAPHICAL ABSTRACT



The dynamics of changes in the prevalence of genes (%) occurring during wastewater treatment processes

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ABSTRACT

The aim of this study was to determine the effect of the activated sludge process on the abundance of anaerobic bacteria of the phylum *Bacteroidetes*, with special emphasis on *Bacteroides fragilis* group (BFG) bacteria, in twelve full-scale wastewater treatment plants. The composition of bacterial phyla and classes in wastewater samples were analyzed by next-generation sequencing. The presence of specific to BFG bacteria genes and the abundance of ARGs and genes encoding class 1 integrase in wastewater samples were determined by qPCR. *Proteobacteria, Firmicutes, Actinobacteria* and *Bacteroidetes* were dominant bacterial phyla in wastewater samples. Next-generation sequencing revealed similar proportions of *Bacteroidia* (< 1.0-8.2 % of all bacteria) in wastewater influents and effluents, which suggest that these microorganisms are not completely eliminated in the activated sludge process. The average copy numbers of specific to BFG bacteria gene, were 10^6, and 10^4 copies in 1 mL of wastewater influents and effluents, respectively. The results revealed a correlation between the abundance of BFG bacteria and BFG-specific genes encoding resistance to antibiotics. The observed changes in the prevalence of BFG-specific genes and ARGs in untreated and treated wastewater indicate that the activated sludge process decreases the number of gene copies in the effluent evacuated to the environment.

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1. Introduction

The presence of antibiotics in the environment promotes the development of antibiotic-resistant bacteria (ARB), which can be attributed to the widespread and often uncontrolled use of antimicrobials in human and veterinary medicine as well as in agriculture (Yang et al., 2014). Antibiotic resistance can be transferred to other bacteria by mobile genetic elements containing antibiotic-resistance genes (ARGs) during horizontal gene transfer (HGT) (Liu et al., 2016; Zhang et al., 2019). The development and spread of ARGs in bacteria poses a serious global problem (Aslam et al., 2018). Most research studies focus on ARB and ARGs in clinical infections, but some studies have provided evidence that environmental factors also play a role in the evolution of drug resistance (Bengtsson-Palme et al., 2018). Wastewater treatment plants (WWTPs) create highly supportive conditions for the development and transfer of ARGs (An et al., 2018; Korzeniewska and Harnisz, 2018; Rizzo et al., 2013). These facilities process household and hospital wastewater which is contaminated with antibiotics as well as sewage from industrial plants, including pharmaceutical plants. Antibiotics are commonly evacuated with sewage to WWTPs around the world (Carvalho and Santos, 2016). Subinhibitory concentrations of antibiotics in wastewater exert continuous selective pressure which, together with high concentrations of diverse bacteria in sewage, in particular in activated sludge tanks, contributes to HGT (Gullberg et al., 2011; Rizzo et al., 2013; Karkman et al., 2018). Hu et al. (2016) demonstrated that the horizontal transfer of resistance genes between species occurs mainly in the bacterial phyla of *Proteobacteria, Firmicutes, Bacteroidetes* and *Actinobacteria.* In their study, these bacterial phyla were significant reservoirs of mobile ARGs and integrons (Hu et al., 2016; Huerta et al., 2013; Snydman et al., 2002). Storteboom et al. (2010) and Osinska et al. (2020) demonstrated that WWTPs significantly influence the presence and spread of ARGs in the environment. In our previous studies, drug-resistant human and animal pathogens were detected in wastewater, including *Aeromonas* spp. (Harnisz and Korzeniewska, 2018), *Enterobacteriaceae* (Osinska et al., 2017; Korzeniewska and Harnisz, 2013) and bacteria of the *Bacteroides fragilis* group (BFG) (Niestepski et al., 2019a, b). These pathogens are evacuated with treated wastewater to the environment where they can pose a threat to human and animal health.

Most research into bacterial communities colonizing wastewater focus on the dominant phyla *Proteobacteria* and *Firmicutes,* including nitrogen-fixing and denitrifying bacteria, as well as potentially pathogenic species such as *Eschericha coli, Enterococcus faecalis* and *Clostridium perfringens* (Liu et al., 2018; Ma et al., 2015; Saarenheimo et al., 2017; Cameron et al., 2019). However, bacteria of the phylum *Bacteroidetes,* which are less abundant in wastewater, in particular in relation to *Proteobacteria*, can play an important role in wastewater. The phylum *Bacteroidetes* is composed of four classes of Gram-negative, non-spore-forming, anaerobic and aerobic bacteria: *Bacteroidia*, *Cytophagia, Flavobacteria* and *Sphingobacteria* (Krieg and Garrity, 2011). *Flavobacteriales* and *Sphingobacteriales* are soil bacteria that are practically undetected in the human and animal digestive tract, with the exception of *Capnocytophaga* spp. and *Sphingobacterium* spp. which colonize the human oral cavity (Rajilic-Stojanovic and de Vos, 2014). *Cytophagia* are ubiquitous in the environment, including in marine ecosystems (Krieg and Garrity, 2011). Obligate anaerobic bacteria belonging to the genus *Bacteroides,* class *Bacteroidia,* as well as *Firmicutes* are the dominant bacteria in the human and animal gastrointestinal tract, and they are highly abundant in raw wastewater (Li et al., 2014; Gomez-Donate et al., 2016; Su et al., 2017). Most bacterial species of the genus *Bacteroides* are essential for healthy gut function in humans and animals, but this genus also contains potentially pathogenic *Bacteroides fragilis* group (BFG) bacteria. Bacterial species of the genus *Bacteroides* are anaerobic microorganisms that are most frequently isolated from infected human tissues (Snydman et al., 2011). They are highly resistant to antibiotics due to natural resistance to aminoglycosides as well as resistance that is acquired during HGT (Husain et al., 2017). Clinical strains of BFG bacteria are highly resistant to many antibiotic classes, including beta-lactams, tetracyclines, macrolides and fluoroquinolones, and they are regarded as reservoirs of ARGs (Kierzkowska et al., 2019; Sydenham et al., 2017; Su et al., 2017). Our previous research demonstrated that environmental BFG strains isolated from wastewater processed by the activated sludge method are also resistant to many antibiotic classes (Niestepski et al., 2019a, b). Bacteria of the genus *Bacteroides* are obligate anaerobes, but they can survive in aquatic environments under oxidative stress conditions for 2–6 days, subject to temperature (Balleste and Blanch, 2010). In our previous study, BFG bacteria were isolated from wastewater influents and effluents, which indicates that these microorganisms are capable of surviving activated sludge treatment (Niestepski et al., 2019a, b).

The influence of wastewater treatment processes on ARB and ARGs has been extensively studied (Zhang et al., 2016; Yuan et al., 2016; Li et al., 2017; Karkman et al., 2018). However, according to the authors' best knowledge, the proportion of *Bacteroidetes* in wastewater and the occurrence of ARGs specific to anaerobic bacteria have never been thoroughly analyzed in wastewater treatment plants deploying the activated sludge method. The present study attempts to fill in this knowledge gap.

In view of the above, the aim of this study was to determine the effect of the activated sludge process on: i) proportion of bacteria of the phylum *Bacteroidetes* in the population of wastewater bacteria, and ii) the abundance of anaerobic bacteria belonging to the *Bacteroides fragilis* group and BFG-specific genes which encode resistance to antibiotics, including class 1 integrons. The correlations between the abundance of BFG bacteria in wastewater and the copy numbers of ARGs encoding resistance of antibiotics and class 1 integrons were also determined.

2. Materials and methods

2.1. Study sites and sampling

Samples of untreated (influent) and treated (effluent) wastewater were collected from 12 wastewater treatment plants (WWTPs) in the Region of Warmia and Mazury, Poland. Grab samples were collected into sterile bottles directly beyond the grate chamber and before wastewater release into a river or a drainage ditch, as described by Korzeniewska and Harnisz (2018). Wastewater samples were collected three times on the same day, and were combined into a composite sample in summer (July). The analyzed WWTPs process various types of wastewater (domestic, hospital and food industry wastewater) using the activated sludge technology. The studied WWTPs were numbered as in a previous study by Korzeniewska and Harnisz (2018). The relevant information is presented in Table S1.

2.2. Extraction of environmental DNA from sewage samples

Wastewater samples consisting of 1000 mL of untreated wastewater and 2000 mL of treated wastewater were collected from 12 WWTPs, and were divided into three parts. Each wastewater sample was filtered separately with the use of 0.2-μm standard mixed cellulose filters with a hydrophobic edge (Merck, Millipore). Spent filters were placed in sterile screw cap tubes (50 mL), and they were stored at −20 °C until analysis. 30 ml of 1 x PBS was added to each tube, and the tubes were shaken (200 rpm, 3 h) at room temperature. The resulting precipitate was placed in 2.0 mL Eppendorf tubes and centrifuged (9000 rpm, 15 min). The Fast DNA SPIN Kit for Soil (MP Biomedicals) was used to extract genomic DNA according to the provided instructions. The extracted DNA was subjected to quantitative and qualitative analysis in the Nanodrop spectrophotometer (NanoDrop® ND-1000, NanoDrop Technologies, Wilmington, DE). Genomic DNA obtained from composite wastewater samples was mixed, and composite DNA samples were stored at −20 °C until analysis.

2.3. 16S rRNA amplicon sequencing and bioinformatic data processing

The proportion of *Bacteroidetes* in the bacterial population was determined by sequencing the V3-V4 hypervariable region of the 16S rRNA gene with the Illumina Miseq v2 Reagent kit (Genomed, Warsaw, Poland) with 2 × 250 bp paired-end reads and primers 341 F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG) and 785R (5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGG TATCTAATCC) designed by Klindworth et al. (2013). The Q5 Hot Start High Fidelity 2x Master Mix was used in the PCR assay according to the manufacturer's protocol. Sequencing reactions were performed with the MiSeq sequencer according to the manufacturer's instructions. Miseq Reporter (MSR) v 2.6 was used for demultiplexing and generating raw fastq files. Illumina metagenomic datasets are available at MG-RAST under accession numbers 4801307.3–4801332.3.

Sequence reads were processed in Mothur v.1.39.3 (Schloss et al., 2009) as previously described by Badri et al. (2013) and Chaparro et al. (2014). The sampling effort was equalized to the depth of the smallest sample (6334 reads), and operational taxonomic units (OTUs) were defined at 97 % sequence identity using the average neighbor algorithm. Chimeric sequences were detected and removed in the UCHIME program (Edgar et al., 2011). Reads were classified in Mothur using the naive Bayesian classifier (Wang et al., 2007). Final taxonomic assignment was based on consensus sequences for each OTU. The sequences were assigned to phylotypes using the phylotype command in Mothur.

2.4. Qualitative analyses of the prevalence of BFG bacteria and resistance and integrase genes

The fragment of the 16S rRNA gene (*bfr* gene and HF183/BacR287 marker) specific to *Bacteroides fragilis* group bacteria was quantified by real-time quantitative PCR (qPCR) in genomic DNA extracted from wastewater samples. The DNA template was used quantify seven genes encoding resistance to four antibiotic classes (beta-lactams (*cfx*A); tetracyclines (*tet*(Q), tet(X); macrolides, lincosamides and streptogramins (*erm*F, *mef*A and *lin*A); fluoroquinolones (*bex*A)) and the integrase gene (*int*I1). All of the tested genes are involved in the antimicrobial resistance of BFG bacteria, and literature data point to a high detection frequency of these ARGs among clinical and environmental BFG strains (Eitel et al., 2013; Kouhsari et al., 2019; Niestepski et al., 2019a). This fact suggests that BFG strains may be a reservoir for these ARGs. The DNA template was used to quantify BFG bacteria and the human-associated marker of *B. dorei* (the *bfr* gene and the HF183/BacR287 marker, respectively) within the 16S rRNA gene as well as the integrase gene (*int*I1). The number of the analyzed gene copies was normalized to 16S rDNA copies of Domain Bacteria. The copy numbers of the above genes were quantified using SYBR Green and qPCR protocols that were optimized based on previously described primers (Table S2). All qPCR reactions were performed in the Roche LightCycler® 480 (Roche Applied Science, Indianapolis, IN, USA) in 20 µL of the reaction mixture containing 1 µl (20 ng) of genomic DNA template. All samples were assayed in triplicate. Standard curves for each gene were created by diluting plasmids carrying the target genes. The curves were used to the measure Ct values and to calculate the copy number of antibiotic resistance genes, taxon-specific gene markers and the integrase gene in samples of untreated and treated wastewater.

2.5. Statistical analysis

Differences in the copy numbers of BFG-specific ARGs and the *int*I1 gene, differences in the composition of bacterial populations in influent and effluent samples, and differences in the structure of bacterial populations colonizing wastewater samples from different WWTPs were determined by one-way ANOVA (test F and Tukey's test; for values with normal distribution) and the Kruskal-Wallis test (KW) (for values with non-normal distribution) (N = 72). The correlations between the technical parameters of WWTPs, number of the analyzed genes and the composition of bacterial samples were determined in Spearman's rank correlation test. Data were processed statistically in Statistica 13.2 software (StatSoft Inc., 1984–2018), and the results were regarded as statistically significant at $\rho < 0.05$. The abundance of the analyzed genes and bacterial populations in the examined wastewater samples was determined by hierarchical cluster analysis with the use of Ward's method. Cluster analyses were performed in the R environment, and the results were presented in heatmaps.

3. Results and discussion

3.1. Structure of bacterial communities

High-throughput next-generation sequencing (NGS) methods support comprehensive analyses of the proportion of *Bacteroidetes* in bacterial communities colonizing wastewater. The relevant information cannot be acquired with the use of conventional culture-based methods (Rodriguez et al., 2015). A total of 934,453 effective sequences clustered in 895 OTUs, ranging from 24,247 to 99,541 sequences per sample, were obtained from influent and effluent samples from 12 WWTPs. The Shannon-Wiener diversity index (H), Simpson's diversity index (1-D) and the species richness estimator (Chao 1) were calculated for each sample, and the results are presented in Table S3. In the statistical analysis, the mean values of the Shannon-Wiener index and Simpson's index were significantly ($p < 0.005$) higher in samples of untreated than treated wastewater. The above findings indicate that untreated wastewater was characterized by higher biological diversity than treated sewage. Similar observations were made by Xue et al. (2019). Shu et al. (2016) also demonstrated higher levels of biodiversity in municipal sewage than in industrial wastewater. In our study, no significant differences in species richness were noted between samples (Chao 1, $p > 0.05$), which suggests that all of the analyzed samples were characterized by similar species diversity.

In the group of the analyzed sequences, 92.8 % were identified at phylum level (15 phyla), and 90.6 % were identified at class level (24 classes). Four dominant phyla were identified in the analyzed wastewater samples: *Proteobacteria* (20.4–53.4 %), *Firmicutes* (16.1–47.6 %), *Actinobacteria* (4.1–40.8 %) and *Bacteroidetes* (2.4–15.7 %) (Fig. 1a). Similar bacterial composition was reported by other authors in hospital wastewater, WWTP wastewater and activated sludge (Caucci et al., 2016; Ferrera and Sanchez, 2016; Kang et al., 2018; Szekeres et al., 2017; Xue et al., 2019; Zhang et al., 2012, 2018). *Bacteroidetes* accounted for 5.1%–15.7% of all bacterial phyla in influents, and from 2.4 % to 12.5 % in effluents. The statistical analysis revealed no significant differences in bacterial abundance between samples of influent and effluent wastewater (KW, $p < 0.18$). *Bacteroidetes* were less abundant that *Fusobacteria* in 11 wastewater samples, in particular in treated wastewater. In three effluent samples, *Bacteroidetes* were more abundant than *Actinobacteria.* The observed differences in the structure of bacterial communities between wastewater samples can probably be attributed to various modifications of the activated sludge technology as well as different types of wastewater processed by the analyzed WWTPs (Shchegolkova et al., 2016).

The Kruskal-Wallis test revealed significant differences only in the relative abundance of *Firmicutes* and *Actinobacteria* ($p < 0.05$) in samples of untreated and treated wastewater. The abundance of the remaining bacterial phyla did not differ significantly between samples of influent and effluent samples. An analysis of relative bacterial abundance at class level revealed that *Gammaproteobacteria* (5.0–41.6 %) and *Betaproteobacteria* (1.3–19.4 %) were the dominant classes in the phylum *Proteobacteria*, whereas *Bacilli* (4.5–37.2 %) and *Clostridia* (3.8–30.1 %) were the most prevalent classes in the phylum *Firmicutes* (Fig. 1b). Similar results were reported by Xue et al. (2019) and Zhao et al. (2017) in samples of municipal WWTP wastewater and river

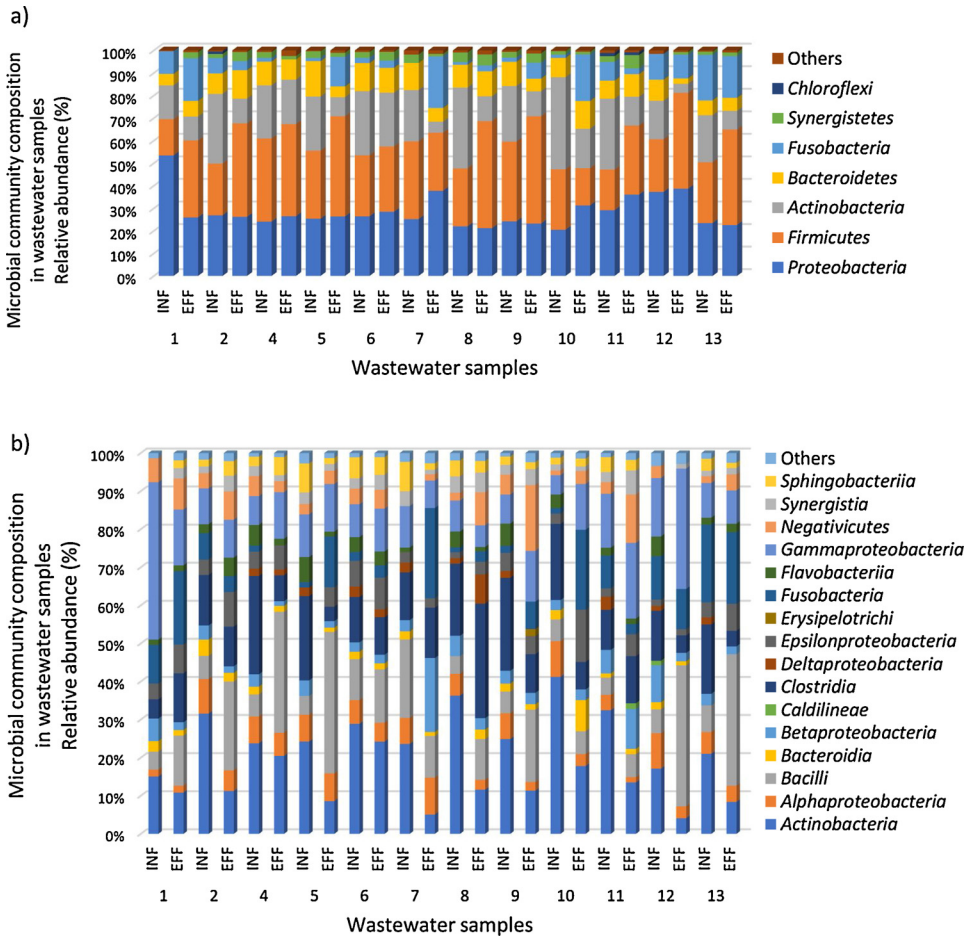

Fig. 1. Microbial community compositions at the phylum (a) and class (b) levels in influent (INF) and effluent (EFF) samples each WWTPs (1–13). Sequences showing a relative abundance of reads < 1.0 % in each samples were grouped into 'Others'.

sediment. *Bacteroidia* was the third most abundant class in the phylum *Bacteroidetes*, and it accounted for < 1.0 % to 8.2 % of bacteria in wastewater samples. The average proportion of *Bacteroidia* did not exceed 2.5 % in most samples. Our results differ considerably from those reported by Fang et al. (2018), where *Bacteroidia* accounted for up to 15 % of all bacterial communities. These discrepancies could be attributed to differences in sewage systems, types of processed wastewater and sampling seasons.

Significant differences in the proportions of *Bacteroidia* were not observed between samples of influent and effluent wastewater (KW, $p < 0.84$). These findings indicate that obligate anaerobes of the class *Bacteroidia* are not completely eliminated by the activated sludge technology and that their percentage in bacterial populations remains similar after wastewater treatment. Similar results were reported by Narciso-da-Rocha et al. (2018).

The Kruskal-Wallis test did not reveal significant differences in the total number of reads allocated to individual taxa, both at phylum and class level, between influent and effluent samples ($p > 0.05$). However, based on the OTU values of 14 dominant classes, the evaluated samples were clustered into three groups with the use of Ward's agglomeration method (Fig. 2). The first group (I) was composed of three wastewater samples (one influent sample and two effluent samples) characterized by the highest values of sequence reads. The second cluster (II) comprised the sequences from 10 samples, mostly influent samples, with moderate OTU values. The third group (III) was composed of sequences from the remaining 11 samples, mostly effluent samples. Group III was characterized by the relatively smallest number of sequence reads. Cluster analysis also revealed that the classes *Bacteroidia, Flavobacteria* and *Sphingobacteria* of the phyla *Bacteroidetes* and *Synergista* formed a homogeneous group. Ding et al. (2019) demonstrated that the above bacterial classes were present in anaerobic wastewater treatment systems where they decomposed complex organic compounds. Municipal wastewater contains a wide variety of microbial communities, including pathogenic species that establish a commensal relationship with humans and animals, and environmental bacteria. The structure of bacterial populations changes during different stages of wastewater treatment. Some species are competitive, unable to survive or reproduce, or form endospores and wait for more favorable conditions (Varela and Manaia, 2013). Differences in local industrial conditions, climate, population burden, demographics and sampling time also have an impact on wastewater characteristics and the composition of bacterial populations present in WWTP systems (Voolaid et al., 2018).

Despite the absence of significant differences in bacterial composition between influent and effluent samples (KW, $p > 0.05$), these samples were clearly separated in Ward's cluster analysis. The distances between clusters were estimated by cluster analysis with the Ward agglomeration method for a detailed interpretation of the results.

3.2. Presence of BFG-specific genes and ARGs in wastewater samples

The number of 16S rRNA gene copies in wastewater samples from 12 WWTPs ranged from 10^{7-10} copies in 1 mL of influent samples to 10^{7-9} copies in 1 mL of effluent samples. Similar values were reported by Caucci et al. (2016).

The abundance of BFG bacteria in wastewater was determined

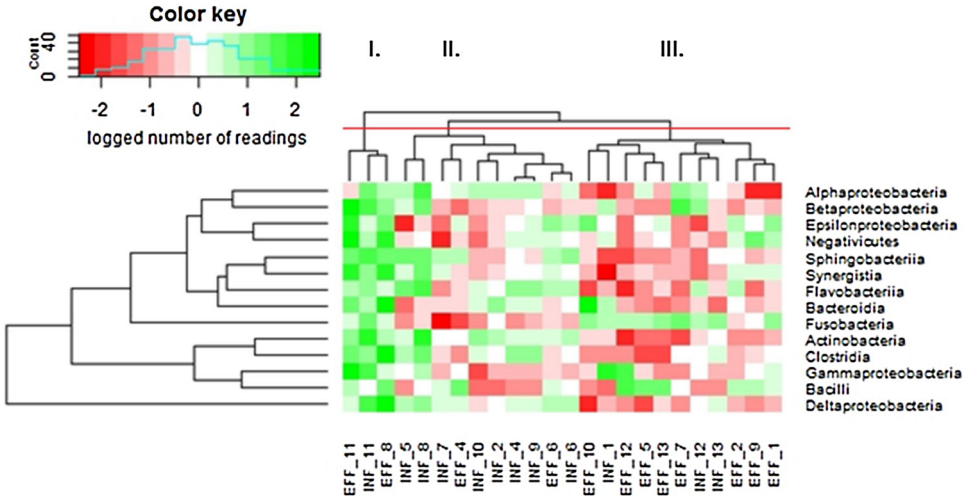

Fig. 2. Heatmap with the logarithmic number of OTU reads at class level in three groups (I-III) of influent (INF) and effluent (EFF) samples from the analyzed WWTPs (1–13).

based on the prevalence of the *bfr* gene and the HF183/BacR287 marker. The *bfr* gene is associated with 10 BFG species (*B. thetaiotaomicron, B. vulgatus, B, fragilis, B. caccae, B. ovatus, B. eggerthii, B. uniformis, B. stercoris, Parabacteroides merdae* and *P. distasonis*), whereas the HF183/BacR287 marker is linked with the presence of *B. dorei* (Liu et al., 2003; Xue et al., 2019). The number of copies of the *bfr* gene and the HF183/BacR287 marker was similar at 10^{2-6} copies in 1 mL of influent wastewater, and 10^{1-4} copies in 1 mL of effluent wastewater. Xue et al. (2019) and Ahmed et al. (2019) also detected the HF183 marker in both influent and effluent samples (10^{0-5} copies/mL wastewater). They demonstrated that despite variations in the abundance of the HF183 marker in wastewater samples from different WWTPs, this marker can be effectively used to detect human fecal contaminants in aquatic environments.

Integrons, in particular class 1 integrons, play a very important role in the development and transmission of ARGs among bacteria (Chamosa et al., 2017; Stalder et al., 2014). The number of *int*I1 gene copies was similar in the analyzed samples of influent and effluent wastewater, ranging from 10^5 to 10^7 copies in 1 mL. Similar values were noted by Zhang et al. (2019) in samples of activated sludge from 18 WWTPs. Korzeniewska and Harnisz (2018) analyzed wastewater from the same WWTPs that were evaluated in this study and found that *int*I2 gene copy numbers were 2–3 orders of magnitude lower. This result indicates that class 1 integrons are increasingly abundant in wastewater, which was also confirmed by other authors (An et al., 2018).

The copy numbers of ARGs in 1 mL of wastewater are presented in Fig. 3. The copy number of the *cfx*A gene encoding resistance to β-lactams was determined at 10^4 to 10^7 in mL of influent samples and at 10^2 to 10^5 copies in 1 mL of effluent samples. Similar values were reported by Fan et al. (2018) in samples of activated sludge from 16 WWTPs. In their study, the most prevalent genes encoding resistance to β-lactams were *cfx*A, *amp*C and *pen*A.

In the group of genes encoding resistance to tetracyclines, *tet*(Q) was characterized by the highest number of copies in both untreated (10^{4-7} copies/mL) and treated wastewater (10^{2-6} copies/ mL). Similar values were reported by Zhang et al. (2019). A comparison of the present results with the findings of Korzeniewska and Harnisz (2018) revealed that the relative number of *tet*(Q) and *tet*(X) gene copies was 1–3 orders of magnitude lower relative to the *tet*(A) gene.

In the group of MLS genes, *erm*F was most abundant in both influent and effluent samples at 10^{5-8} copies/mL and 10^{6-7} copies/mL, respectively. The total relative copy numbers of *erm*F, *mef*A and *lin*A ranged from 1.5×10^{-3} do 4.5×10^{-2} copies/16S rRNA in untreated wastewater, and from 3.8×10^{-4} do 7.4×10^{-2} copies/16S rRNA in treated wastewater. These values are similar to the copy numbers of the *mef*A gene detected in hospital wastewater (Szekeres et al., 2017) and the copy numbers of the *erm*F gene in a municipal WWTP with an activated sludge process (Yang et al., 2014).

The copy number of the *bex*A gene which encodes resistance to fluoroquinolones ranged from 10^1 to 10^4 copies/mL in untreated wastewater, and it was one order of magnitude lower in treated wastewater. The *bex*A gene and other genes synthesizing multidrug efflux proteins are relatively prevalent in wastewater. In a study by Yang et al. (2014), their abundance was determined at 10^{6-7} copies/mL of wastewater.

In the current study, genes encoding resistance to MLS drugs were encountered most frequently in the group of the analyzed ARGs. According to Graham et al. (2011), the relative abundance of ARGs in strongly polluted environments can reach or exceed 10^{-4} copies/16S rRNA copies. This observation suggests that the wastewater samples analyzed in this study were strongly contaminated with ARGs. The abundance of ARGs in treated wastewater was significantly reduced relative to the inflow, mostly likely due to their transfer together with bacteria to sewage sludge. Excess sewage sludge is separated from wastewater and removed from the wastewater treatment process. This hypothesis was confirmed by Ju et al. (2016) and Mao et al. (2015) who noted great abundance of ARGs in sewage sludge. The abundance of the evaluated genes is presented in Figures S1 and S2.

The correlations between the abundance of ARGs in the tested wastewater samples were determined by Ward's method, and the results are presented in Fig. 4. The cluster analysis produced three groups. Group I was composed of 5 samples, mostly influent samples, with the highest ARG copy numbers. Group II consisted of 8 samples, mostly effluent samples, and it was characterized by the lowest prevalence of ARGs. Group III was composed of 11 samples with moderate and similar abundance of ARGs. The last group was further divided into two subclusters. The first sub-cluster contained 4 effluent samples, and the second sub-cluster was composed of 7 influent samples. An analysis of the heatmaps illustrating the correlations between OTUs at class level (Fig. 2) and the abundance of ARGs (Fig. 4) indicates that influent samples and effluent samples tend to form separate clusters.

3.3. The effect of the activated sludge process on the abundance of BFG bacteria and ARGs

Wastewater is treated by the activated sludge method in all analyzed WWTPs, which were divided into four groups based on

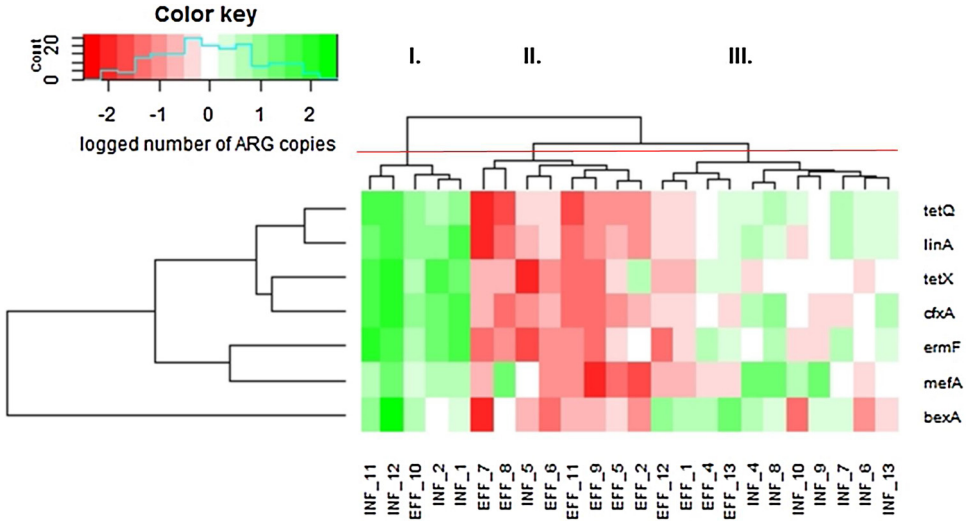

Fig. 3. Prevalence of drug resistance genes (number of copies/1 mL) in wastewater (INF – influent; EFF – effluent; 1-13 – number of the analyzed WWTPs, I – III – group number).

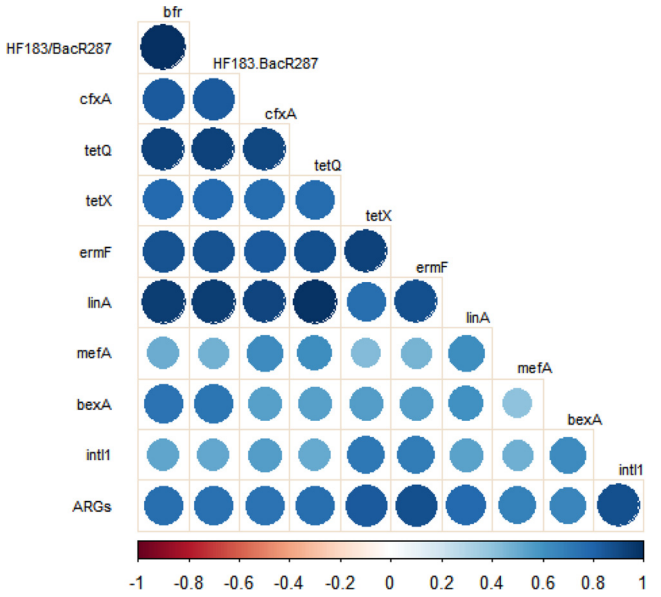

Fig. 4. Spearman's rank correlation coefficient between concentration of ARGs (copies number/mL) ($p < 0.05$).
Positive correlations are displayed in blue and negative correlations in red color. The color intensity and the size of the circle are proportional to the correlation coefficients.

modifications of the treatment technology (Table S1). Tukey's test (one-way ANOVA) was performed to verify the significance of differences between the groups of WWTPs based on operational parameters such as average processing capacity, hydraulic retention time and sludge age (SA), as well as biochemical oxygen demand (BOD) and chemical oxygen demand (COD) in wastewater samples. No significant differences were found between the analyzed groups of WWTPs (Table S4). Therefore, the abundance of ARGs and the structure of bacterial populations colonizing wastewater samples were analyzed collectively for all WWTPs.

The wastewater treatment processes in the evaluated WWTPs exerted varied effects on the copy numbers of 16S rRNA, *bfr*, HF183/BacR287 and ARGs in treated wastewater. Detailed information about changes in gene copy numbers after wastewater treatment is presented in Table 1.

In 6 out of the 12 studied WWTPs, the treatment process decreased the number of 16S rRNA gene copies by 77.4–97.1 %. A 26.9 % reduction in 16S rRNA gene abundance was noted in one WWTP. In 4 WWTPs, the number of 16S rRNA gene copies increased significantly in treated wastewater relative to untreated sewage. The influence of the activated sludge process on the abundance of the 16S rRNA gene in treated wastewater cannot be clearly elucidated based on the obtained results. The effectiveness of the activated sludge technology has been broadly discussed in the literature. In some studies, bacterial populations were effectively reduced by the activated sludge process (Oliveira et al., 2016), whereas other authors reported an increase in the number of 16S rRNA gene copies in treated wastewater (Rafraf et al., 2016). According to Koivunen et al. (2003), the effectiveness of wastewater treatment processes can differ due to variations in plant performance or the type of processed wastewater.

The rate of changes in the abundance of the *bfr* gene and the HF183/BacR287 marker in wastewater samples was nearly identical (correlation close to 1.0). This result points to close correlations between BFG bacterial species detected based on the *bfr* gene and the HF183/BacR287 marker. In 9 out of the 12 examined WWTPs, the prevalence of the *bfr* gene was significantly reduced by 57.6 % to nearly 99.99 % after wastewater treatment. The number of HF183 marker copies was also significantly reduced in treated wastewater in a study by Xue et al. (2019). The cited authors observed that the extent to which the above gene is transferred to treated wastewater is considerably influenced by the applied treatment process.

In 8 out of the 12 analyzed WWTPs, the copy numbers of the *int*I1 gene encoding class 1 integrons decreased significantly by 21.6%–98.3% in treated wastewater. In contrast, the abundance of the *int*I1 gene in treated wastewater increased significantly in the remaining 4 WWTPs. Korzeniewska and Harnisz (2018) reported a decrease in the number of *int*I2 gene copies in wastewater samples collected from all evaluated WWTPs. In the present study, the copy numbers of the *int*I1 gene increased in WWTPs where the abundance of ARGs tended to increase after wastewater treatment.

The number of *cfx*A gene copies was reduced by 83.1%–99.998% in 10 out of the 12 evaluated WWTPs. In the remaining 2 WWTPs, the abundance of the *cfx*A gene increased significantly in effluent samples. In other studies, genes encoding resistance to beta-lactams were also effectively removed from influent wastewater (Yang et al., 2014).

In 10 out of the 12 examined WWTPs, the number of *tet*(Q) gene copies was reduced by 80.2%–99.988%. A significant increase in *tet*(Q) abundance was noted in effluent samples in WWTP No. 10. In 8 out of the 12 analyzed WWTPs, the prevalence of *tet*(X) gene decreased by

Table 1
The dynamics of changes in the prevalence of genes (%) occurring during wastewater treatment processes.

WWTPs	16S rRNA	*bfr*	HF183/ BacR287	*int*I1	*cfx*A	*tet*(Q)	*tet*(X)	*erm*F	*lin*A	*mef*A	*bex*A
1	93.636*	86.849*	86.866 *	94.079 *	99.816 *	96.757 *	97.808 *	96.818 *	99.865 *	95.508 *	−7.547
2	13.340	97.380*	97.369 *	21.591 *	99.898 *	99.426 *	78.937 *	90.255 *	99.854 *	99.235 *	90.236 *
4	−169.039*	61.833*	61.817 *	−388.056 *	83.128 *	80.183 *	−207.014 *	−244.269 *	89.511 *	96.849 *	−24.293
5	−1644.408*	−36.158	−36.258	−811.715 *	−86.415 *	86.673 *	−2922.014 *	−899.000 *	20.697 *	94.848 *	−140.417
6	77.964*	79.426*	79.423*	64.422 *	83.602 *	85.639 *	75.677 *	82.681 *	88.307 *	80.548 *	54.167 *
7	−68.138*	99.966*	99.966*	90.020 *	69.640 *	99.978 *	74.240 *	96.978 *	99.989 *	84.513 *	98.739 *
8	81.180*	99.076*	99.076*	37.280 *	99.926 *	99.903 *	70.291 *	95.896 *	99.806 *	−24.403 *	53.038
9	77.350*	57.573*	57.602*	59.130 *	97.619 *	95.124 *	91.520 *	89.825 *	98.345 *	99.945 *	83.285 *
10	−225.463*	−2142.788*	−2139.581*	−722.531 *	−2875.305 *	−1417.088 *	−228.586 *	−899.000 *	−6550.711 *	73.301 *	−6397.838 *
11	96.429*	99.988*	99.941*	93.723 *	99.998 *	99.998 *	99.701 *	99.718 *	99.995 *	97.006 *	97.785 *
12	97.125*	99.943*	99.943*	98.275 *	99.946 *	99.869 *	99.375 *	99.590 *	99.928 *	98.614 *	96.575 *
13	26.849*	−241.967*	−242.594*	−47.131 *	96.475 *	7.325	−36.247	18.286 *	47.824 *	65.252 *	−3777.329

* denotes a statistically significant difference ($p < 0.05$).

70.3%–99.7%. The number of *tet*(X) gene copies increased after wastewater treatment in 3 WWTPs. In WWTP No. 13, no significant differences were found in the abundance of *tet*(Q) and *tet*(X) genes between influent and effluent samples. Chen and Zhang (2013a, b), Liu et al. (2013) and Yang et al. (2014) reported a decrease in the abundance of genes encoding resistance to tetracyclines after wastewater treatment, whereas Korzeniewska and Harnisz (2018) did not observe significant differences in the number of *tet*(Q) and *tet*(X) gene copies between untreated and treated wastewater. Detailed monitoring studies are needed to elucidate the effect of wastewater treatment processes on the abundance of genes encoding resistance to tetracyclines.

The extent to which MLS genes (encoding resistance to macrolides, lincosamides and streptogramins) were eliminated during wastewater treatment varied across the analyzed WWTPs. The number of *mef*A and *lin*A gene copies decreased by 47.8–99.995 % after wastewater treatment in 11 WWTPs, whereas the abundance of the *erm*F gene was reduced by 18.3%–99.7% in 9 WWTPs. The results noted in all examined WWTPs indicate that the activated sludge technology supports the elimination of MLS genes. Similar observations were made by Nolvak et al. (2013); Mao et al. (2015) and Yang et al. (2014).

The number of *bex*A gene copies decreased significantly after wastewater treatment in 6 out of the 12 evaluated WWTPs. The prevalence of the *bex*A gene decreased by 54.2%–98.7%. A significant increase in the abundance of the *bex*A gene in untreated wastewater was noted in 2 WWTPs, but in the remaining 4 WWTPs, significant differences in the number of *bex*A gene copies were not observed between influent and effluent samples. These results suggest that the activated sludge technology does not effectively eliminate the *bex*A gene from wastewater. The above observation gives cause for concern because the *bex*A gene encodes the synthesis of multi-antimicrobial extrusion proteins (MATE) responsible for, among others, resistance to fluoroquinolones (Pumbwe et al., 2006; Sarvari et al., 2018). Very little is known about the fate of MATE-encoding genes during wastewater processing, and further research is required to elucidate this phenomenon.

In one of the analyzed WWTPs with the activated sludge technology, the abundance of nearly all of the studied genes (11 out of 12, 91.67 %,), including the 16S rRNA gene, BFG-specific genes, *int*I1 and six ARGs, increased after wastewater treatment. This WWTP operated a sequencing batch reactor (SBR) with the longest SA (44 days) in the group of the studied plants (Table S1). According to many authors, long SA contributes to the growth and accumulation of microorganisms that degrade antibiotics and act as reservoirs of ARGs and mobile genetic elements (Shahzad et al., 2015; Wang et al., 2019; Zeng et al., 2013). However, two WWTPs with enhanced removal of biogenic substances eliminated more than 93 % of all studied genes. These WWTPs were characterized by the shortest SA and the shortest hydraulic retention time (HRT). The present study confirmed the presence of positive correlations between SA vs. the occurrence of *bfr*, *tet*(Q), *erm*F and *mef*A genes and the copy numbers of ARGs in treated wastewater ($r = 0.82$-0.78, $p < 0.05$) (Table S5). The effects of HRT and SA on the rate of ARG removal from wastewater have been discussed in more detail by Korzeniewska and Harnisz (2018) and (Nnadozie et al., 2017).

3.4. Correlations between the composition of microbial communities, the prevalence of the bfr gene, HF183/BacR287 marker and ARGs in wastewater

The correlations between the composition of microbial communities in wastewater were determined by MiSeq Illumina sequencing. An analysis of microbial composition in wastewater samples at phylum level revealed moderate correlations between the abundance of *Bacteroidetes* vs. *Proteobacteria* ($r = 0.460$, $p < 0.05$) and *Actinobacteria* ($r = 0.763$, $p < 0.05$) and *Bacteroidetes* ($r = 0.667$, $p < 0.05$), and between *Synergistetes* vs. *Firmicutes, Actinobacteria* and ($r = 0.550$-0.696, $p < 0.05$) (Table S6). The abundance of *Bacteroidia* was also positively correlated with *Epsilonproteobacteria* ($r = 0.474$, $p < 0.05$) (Table S7). *Alphaproteobacteria*, *Deltaproteobacteria, Clostridia, Actinobacteria* and *Synergistia* were most highly and significantly correlated with the remaining bacterial classes ($r = -0.407$ – 0.786, $p < 0.05$).

The changes in bacterial composition determined by MiSeq Illumina sequencing as well as the changes in gene abundance determined by qPCR before and after wastewater treatment can be burdened with error. The MiSeq system processes massive amounts of data, and it can produce sequencing artefacts (Unno, 2015). For this reason, in the presented study, the correlations between the prevalence of BFG bacteria and BFG-specific ARGs were determined only based on the results of qPCR assays.

The results of the correlation analysis examining the relationships between the evaluated genes and total ARGs are presented in Fig. 4 and Table S8. A significant positive correlation was noted between the concentrations of all analyzed genes ($r = 0.409$-0.999, $p < 0.05$). The values of Spearman's correlation coefficient demonstrated that BFG-specific fragments of the 16S rRNA gene (*bfr*, HF183/BacR287) and the *int*I1 gene were highly correlated with every ARG and with ΣARGs ($r = 0.477$-0.942, $p < 0.05$) (Fig. 4). The strongest correlations were noted for *cfx*A, *tet*(Q), *erm*F and *lin*A genes ($r > 0.8$, $p < 0.05$). These results indicate that BFG-specific genes have a moderate capacity for encoding antibiotic resistance on class 1 integrons which transfer ARGs to other bacteria. Osinska et al. (2019); Su et al. (2018) and Zhang et al. (2019) also reported high correlations between the prevalence of bacteria and the concentrations of ARGs in wastewater samples. It is widely recognized that wastewater from municipal WWTPs serve as important reservoirs for resistance genes located on mobile genetic elements, such as integron-embedded ARGs (Mokracka et al., 2012; Ramsden et al., 2010). A high correlation between integrons and ARGs in treated

wastewater suggests the presence of drug-resistant bacteria, including members of the BFG, which may contribute to environmental pollution.

4. Conclusions

The effect of the activated sludge process on the prevalence of *Bacteroidetes* bacteria in wastewater, and the abundance of genetic markers specific to BFG bacteria, genes encoding class 1 integrons, and BFG-specific ARGs was investigated in the present study. *Bacteroidetes* was one of the dominant bacterial phyla in the analyzed wastewater samples. An analysis of the prevalence of the class *Bacteroidia* relative to other bacterial groups in wastewater influents and effluents revealed that these anaerobic bacteria are not completely eliminated by the activated sludge technology and that their percentage in bacterial populations remains similar after wastewater treatment. The observed changes in the occurrence frequency of the analyzed genes after wastewater treatment suggest that short sludge retention time and short hydraulic retention time contribute to a decrease in gene copy numbers, including BFG-specific genes, ARGs and *int*I1. Despite the above, the abundance of above genes in effluent wastewater was high enough to strongly contaminate the environment. The close links between the abundance of BFG-specific fragments of the 16S rRNA gene, ARG markers and the integrase gene could indicate that BFG bacteria can harbor ARGs and transfer them to other bacteria. Further research is needed to elucidate the effect of wastewater treatment on the prevalence of the phylum *Bacteroidetes* in bacterial populations and the abundance of ARGs and markers specific to *Bacteroides fragilis* group bacteria in wastewater.

CRediT authorship contribution statement

Niestępski Sebastian: Writing - original draft, Writing - review & editing, Investigation, Resources. **Harnisz Monika:** Conceptualization, Methodology, Supervision, Project administration. **Ciesielski Sławomir:** Software, Data curation. **Korzeniewska Ewa:** Conceptualization, Methodology. **Osińska Adriana:** Visualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jhazmat.2020.122544.

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