Wastewater treatment plants (WWTPs) that rely on a mechanical biological treatment system constitute a major source of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs), which are evacuated to rivers with effluents and therefore can spread into the environment. The genetic determinants of antibiotic resistance can be transmitted, with the bioaerosol emitted during wastewater mixing and aeration in activated sludge bioreactors, to the upper respiratory tracts of WWTP employees, thus posing a potential health threat and facilitating the spread of ARGs by horizontal gene transfer (HGT). The aim of the study was to determine whether conventional WWTPs contribute to the transmission of potentially pathogenic microorganisms and the genetic determinants of antibiotic resistance to the aquatic environment and the upper respiratory tracts of WWTP employees. The study whose results constituted the basis for this doctoral dissertation was conducted in four stages, and the findings were published in four original research articles.

The aim of the first stage of the study was to analyze the prevalence of genes encoding resistance to beta-lactam antibiotics in samples of river water, sewage sludge and wastewater in each stage of treatment involving the activated sludge process in two WWTPs. The samples were collected in two seasons (summer, fall). The presence of beta-lactamase genes (*bla*$_{\mathrm{TEM}}$, *bla*$_{\mathrm{OXA}}$, *bla*$_{\mathrm{SHV}}$) in the DNA isolated from the analyzed samples was determined by standard polymerase chain reaction (PCR) The *bla*$_{\mathrm{TEM}}$, *bla*$_{\mathrm{OXA}}$ and *bla*$_{\mathrm{SHV}}$ genes were identified in all wastewater samples from the WWTP located in a forested area, in both seasons. The *bla*$_{\mathrm{TEM}}$ and *bla*$_{\mathrm{OXA}}$ genes were detected in wastewater samples from the WWTP located in an urbanized area, in both seasons. The *bla*$_{\mathrm{SHV}}$ gene was found in wastewater samples collected in fall during all stages of the treatment process, whereas it was not detected in treated wastewater samples collected in summer. The *bla*$_{\mathrm{TEM}}$ and *bla*$_{\mathrm{SHV}}$ genes were identified in river water sampled upstream from the WWTP located in a forested area, in both seasons. The *bla*$_{\mathrm{OXA}}$ gene was detected in river water sampled downstream from this WWTP in summer, whereas the *bla*$_{\mathrm{SHV}}$ gene was not found. All analyzed genes were identified in river water sampled downstream from the WWTP located in a forested area in fall. All analyzed genes were also detected in river water sampled upstream from the WWTP situated in an urbanized area in fall, whereas the *bla*$_{\mathrm{SHV}}$ gene was not found in summer. In both seasons, river water sampled downstream from this WWTP contained the *bla*$_{\mathrm{TEM}}$ and *bla*$_{\mathrm{OXA}}$ genes, whereas the *bla*$_{\mathrm{SHV}}$ gene was not present. The above findings may suggest that ARGs are not completely removed during the wastewater treatment process, which poses a potential risk of their transmission to the aquatic environment.

The aim of the second stage of the study was to analyze the prevalence of drug-resistant and virulent *Staphylococcus spp.* strains in river water samples, wastewater samples and upper respiratory tract specimens collected from WWTP employees. Samples of river water collected upstream and downstream from the wastewater discharge point, untreated wastewater, wastewater from an activated sludge bioreactor, treated wastewater as well as nasal and throat swabs collected from WWTP employees and from the control group were analyzed. Samples were collected three times during the year (winter, summer, fall). Genomic DNA was isolated from the samples, and the nuclease (*nuc*) gene and ARGs were identified. The samples were also used to culture *Staphylococcus spp.* bacteria; as a result, a total of 249 bacterial strains were obtained. The strains were analyzed for the presence of the *nuc* gene, virulence genes and ARGs by PCR. In nearly all analyzed samples, at least one *Staphylococcus spp*. strain harbored the *nuc* gene. The methicillin resistance gene (*mec*A) was not identified in any of the analyzed strains obtained from wastewater, river water or control group swabs. This gene was detected in strains isolated from upper respiratory tract specimens collected from WWTP employees. The vancomycin resistance gene (*van*A) was most prevalent (49%) in strains obtained from wastewater from an activated sludge bioreactor. *hla* was the most prevalent virulence gene, followed by *pvl* and *sas*X, whereas *tst1* and *sec* were detected least frequently. In the next step of this stage of the study, a phylogenetic analysis of all *Staphylococcus spp.* strains was performed. Dendrograms were developed based on ERIC-PCR profiles and the prevalence of the analyzed genes to determine the relatedness between the strains isolated from treated wastewater and river water collected downstream from the wastewater discharge point, and between the strains isolated from wastewater and swabs collected from WWTP employees. A high degree of genetic similarity was observed between the strains obtained from wastewater from an activated sludge bioreactor and those isolated from WWTP employees. The above findings may suggest that these microorganisms can be potentially transmitted, with the bioaerosol emitted during wastewater mixing and aeration in activated sludge bioreactors, to the upper respiratory tracts of WWTP employees. In the last step of this stage of the study, a qPCR assay of environmental DNA isolated from the tested samples was performed to determine the concentrations of *nuc*, *mec*A, *van*A and *qac*A/B genes. The highest concentration of the *nuc* gene was noted in untreated wastewater (7.76×10^3 to 5.87×10^4 gene copies/mL) and in wastewater from an activated sludge bioreactor (2.72×10^3 to 1.82×10^4 gene copies/mL). The *mec*A gene was identified in untreated wastewater samples in all seasons, in the concentration of 3.05×10^2 to 2.68×10^3 gene copies/mL. The *van*A gene was detected in untreated wastewater samples in all seasons (8.28×10^5 gene copies/mL on average), whereas

the *qac*A/B gene was identified in all samples excluding those from an activated sludge bioreactor (1.02×10^2 to 6.97×10^5 gene copies/mL). The study demonstrated that WWTPs are a source of ARGs, and effluents discharged to rivers can contribute to their spread into the environment.

The aim of the third stage of the study was to determine the extent of potential transmission of selected pollutants evacuated with effluents to rivers and with the bioaerosol emitted during wastewater mixing and aeration to the upper respiratory tracts of WWTP employees. Samples of river water collected upstream and downstream from the wastewater discharge point (URW and DRW, respectively), untreated wastewater, wastewater from an activated sludge bioreactor as well as nasal and throat swabs collected from WWTP employees and from the control group were analyzed. Samples were collected in three different seasons (winter, summer, fall) to account for potential differences in antibiotic consumption throughout the year. ARGs, genes encoding resistance to heavy metals (HMs) and integrase genes were identified in the genetic material by qPCR. The values of selected physicochemical parameters, and the concentrations of HMs and popular antibiotics were also determined in the samples. All of the 17 analyzed ARGs were detected in at least one sample of wastewater or river water. The highest concentrations of genes encoding resistance of beta-lactams and MLS antibiotics were noted in wastewater samples. The concentrations of most ARGs decreased during successive stages of wastewater treatment. River water samples contained 13 out of the 17 analyzed ARGs; the concentrations of all ARGs were higher in DRW samples than in URW samples. *int*I1 was the most prevalent integrase gene, and *cnr*A was the only identified representative of genes encoding resistance to HMs. In the next step of this stage of the study, the relative concentrations (gene copies/16S rRNA copies) of five selected ARGs (bla_{TEM}, *Amp*C, *sul*1, *erm*F and *mef*A) and the class 1 integron-integrase gene (*int*I1) were determined in the DNA isolated from swabs collected from WWTP employees and control group members (not employed in WWTPs). The concentrations of most analyzed genes were higher in the DNA isolated from swabs collected from WWTP employees than in control group swabs. *erm*F (2.62×10^{-2}–7.45×10^{-3} gene copies/16S rRNA copies) and *mef*A (1.54×10^{-1}–4.84×10^{-2} gene copies/16S rRNA copies) were most prevalent in throat swabs, whereas *int*I1 (5.81–7.72×10^{-1} gene copies/16S rRNA copies) and *sul*1 (1.74–2.62×10^{-2} gene copies/16S rRNA copies) were most prevalent in nasal swabs. All analyzed genes present in the DNA isolated from swabs collected from WWTP employees were also identified in the DNA isolated from wastewater from an activated sludge bioreactor. Employees of WWTPs that rely on a mechanical biological

treatment system, characterized by intense bioaerosol emissions, are exposed to the risk of ARG transmission from wastewater to the upper respiratory tract. An analysis of Spearman's rank correlation coefficients revealed numerous significant positive correlations between the values of selected physical parameters and the concentrations of the analyzed ARGs. The study demonstrated that environmental physicochemical parameters can induce changes in the prevalence and abundance of ARGs in water bodies and affect their transmission in the environment. The presence of antibiotic residues and transformation products (TPs) is an important consideration. It was found that samples of untreated and treated wastewater contained 13 of the 16 analyzed antibiotics, untreated wastewater samples contained the highest concentrations of trimethoprim (2.45–1643.74 ng/L), ciprofloxacin (92.16–306.79 ng/L), clarithromycin (69.30–401.79 ng/L), sulfamethoxazole (118.82–160.27 ng/L) and doxycycline (16.44–97.91 ng/L). Treated wastewater samples contained all antibiotics identified in untreated wastewater samples. The concentrations of most analyzed antibiotics were higher in river water samples collected downstream than upstream from the wastewater discharge point. The effectiveness of drug removal during the technological process varied depending on antibiotic type and season. Complete removal of levofloxacin and a high reduction in the concentrations of sulfadiazine (87.7–94%), sulfamethoxazole (81.8–84%) and ciprofloxacin (79.6–82.6%) were observed in winter and summer. The concentrations of all HMs in untreated wastewater samples remained within the reference ranges (Regulation of the Minister of Maritime Economy and Inland Navigation, MEIN 2019), whereas the zinc content of wastewater from an activated sludge bioreactor (1.47–2.39 mg/L) exceeded the threshold limit (≤1 mg/L) in all samples. Wastewater treatments processes in the analyzed WWTP lead to a considerable decrease in the content of HMs in treated wastewater, which could result from their accumulation in sewage sludge removed from the WWTP. Significant positive correlations were noted between the concentrations of class 1, 2 and 3 integron-integrase genes and a wide array of ARGs, between the concentrations of the *cnr*A gene and 12 ARGs, and between the concentrations of integrase genes and antibiotics. Significant positive correlations ($r = 0.60–0.96$) were also found between the concentrations of ARGs and HMs, in particular zinc and copper. The results of this study confirmed that integrons play an important role in the dissemination of antibiotic resistance.

The aim of the fourth stage of the study was to determine whether 1) WWTPs that rely on the activated sludge technology contribute to the transmission of ARGs with the bioaerosol produced during wastewater treatment to the upper respiratory tracts of WWTP employees, and

2) ARGs can reach surface water bodies with effluents discharged to rivers. Samples of river water and wastewater, and specimens collected from WWTP employees were analyzed by metagenomic next-generation sequencing. The prevalence of ARGs and mobile genetic elements (MGEs), and the taxonomic composition of microorganisms were determined in the samples. Taxonomic diversity was highest in untreated wastewater samples, and lowest in treated wastewater samples. The predominant bacterial phyla were Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes. URW samples were characterized by lower biodiversity than DRW samples. Swabs collected from WWTP employees were characterized by similar taxonomic composition of microorganisms, and Firmicutes, Actinobacteria and Bacteroidetes were the dominant phyla. The relative abundance of ARGs ranged from 2.88×10^{-5} gene copies /16S rRNA to 1.27 gene copies/16S rRNA copies in UWW samples, from 1.3×10^{-4} gene copies /16S rRNA copies to 9.0×10^{-2} gene copies/16S rRNA copies in AS samples, and from 2.62×10^{-5} gene copies/16S rRNA copies to 7.3×10^{-1} gene copies/16S rRNA copies in TWW samples. The predominant ARG types were multidrug resistance genes, followed by genes encoding resistance to MLS antibiotics, beta-lactams and tetracyclines. The abundance of ARGs in water samples ranged from 1.84×10^{-5} gene copies/16S rRNA copies to 2.244 gene copies/16S rRNA copies in URW samples, and from 9.98×10^{-6} to 1.83 gene copies/16S rRNA copies in DRW samples. Water samples were characterized by the highest concentrations of multidrug resistance genes, followed by genes encoding resistance to beta-lactams, MLS antibiotics and aminoglycosides. The diversity of ARGs was higher in DRW samples than in URW samples. The concentrations of ARGs ranged from 3.96×10^{-5} gene copies/16S rRNA copies to 4.61×10^{-1} gene copies/16S rRNA copies in nasal swabs, and from 1.38×10^{-5} gene copies/16S rRNA copies to 1.57×10^{-1} gene copies/16S rRNA copies in throat swabs. Nasal swabs were characterized by the highest concentrations of genes encoding resistance to MLS antibiotics, aminoglycosides and chloramphenicol, and throat swabs – by the highest concentrations of genes conferring resistance to beta-lactams, tetracyclines and MLS antibiotics. The identified families of insertion sequences (IS) were most prevalent in untreated wastewater, and IS3, IS4 and IS21 were most abundant. The abundance of the examined IS families was higher in river water sampled downstream than upstream from the discharge point, and IS3, IS4 and IS5 were most prevalent. The proportions of IS families in nasal and throat samples were similar, with a predominance of IS3, ISL3 and IS30. An analysis of ARGs in terms of the transmission of antimicrobial resistance determinants and microorganisms with effluents discharged to the river revealed that the relative abundance of nearly all analyzed ARGs was higher in DRW samples than in URW samples. Moreover, DRW samples contained

ARGs that were not detected in URW samples. In both URW and DRW samples, the most abundant ARGs were *bac*A, *bcr*A, *emr*E, *mex*D, *aph(3")-I*, *sul*1, *tet*Q and *erm*F. It was also found that eight the 10 examined IS families were more abundant in DRW samples than in URW samples. An analysis of correlation coefficients demonstrated that the identified bacterial phyla were correlated with the concentrations of the analyzed ARGs and IS families. An analysis of data in terms of the transmission of microorganisms and antimicrobial resistance determinants to the upper respiratory tracts of WWTP employees revealed that the concentrations of some ARGs in nasal and throat swabs were similar to those in wastewater samples. *erm*C, *erm*X, *ant(2")-I* and *sul*1 predominated in nasal swabs, whereas *cfx*A2, *tet*M, *tet*Q and *erm*B predominated in throat samples. Significant positive correlations were noted between the concentrations of ARGs and the abundance of microbial phyla, which were identified in swabs collected from WWTP employees and wastewater. Swabs collected from WWTP employees contained IS which are not characteristic of the human microbiome, and which were detected in untreated wastewater samples and wastewater samples from an activated sludge bioreactor. The above findings indicate that effluent discharge increases the pool of the genetic determinants of antibiotic resistance in surface water bodies, and point to the risk of their transmission to the upper respiratory tracts of WWTP employees. The results of this study indicate that antibiotic-resistant microorganisms and antimicrobial resistance determinants can be transmitted, with bioaerosols emitted during wastewater treatment, to the upper respiratory tracts of WWTP employees, which poses health and epidemiological threats. The study highlights the need for the appropriate safety procedures and preventive measures, including personal protective equipment for WWTP employees, and for the development and implementation of new wastewater treatment technologies to minimize bioaerosol emissions. The study also demonstrated that numerous antibiotic-resistant and potentially pathogenic microorganisms, antimicrobial resistance determinants and chemical contaminants may be evacuated to rivers with treated wastewater.