UNIVERSITY OF WARMIA AND MAZURY IN OLSZTYN

FACULTY OF BIOLOGY AND BIOTECHNOLOGY



DOCTORAL DISSERTATION

Wiktor Zygmunt Zieliński, MSc

Wastewater treatment plants as a source of antibiotic resistance transmission – an epidemiological threat to the aquatic environment and plant employees

Written under the guidance of

Professor Ewa Korzeniewska, Dr. Habil., Eng.

Ewa Felis, Dr. Habil., Eng., Professor at the Silesian University of Technology

Olsztyn 2022

UNIWERSYTET WARMIŃSKO-MAZURSKI W OLSZTYNIE

WYDZIAŁ BIOLOGII I BIOTECHNOLOGII

ROZPRAWA DOKTORSKA

mgr Wiktor Zygmunt Zieliński

Oczyszczalnie ścieków jako źródło transmisji antybiotykooporności – zagrożenie epidemiologiczne dla środowiska wodnego i pracowników zakładu

Pod kierunkiem

prof dr hab. inż. Ewy Korzeniewskiej

dr hab. inż. Ewy Felis, profesor Politechniki Śląskiej

Olsztyn 2022

I would like to express my sincere thanks to:

Professor Ewa Korzeniewska, Dr. Habil., Eng.

for scientific supervision, substantive support, the possibility of carrying out a doctoral dissertation

Ewa Felis, Dr. Habil., Eng., Professor at the Silesian University of Technology

for the possibility of a research internship, scientific consultations, support and kindness

Monika Harnisz, Dr. Habil., Eng., Professor at the University of Warmia and Mazury

for scientific consultation, support and kindness

Employees and PhD students of the Department of Water Protection Engineering and Environmental Microbiology

for support, nice working atmosphere and time spent together

The candidate's doctoral research was presented in the following published articles:

1. **Zieliński W**., Buta M., Hubeny J., Korzeniewska E., Harnisz M., Nowrotek M., Płaza G. 2019. Prevalence of Beta Lactamases Genes in Sewage and Sludge Treated in Mechanical-Biological Wastewater Treatment Plants. Journal of Ecological Engineering 20(9), 80–86. https://doi.org/10.12911/22998993/112506. Ministry of Education and Science scoring$_{2021}$: 40; PhD student participation: 51%
2. **Zieliński W**., Korzeniewska E., Harnisz M., Hubeny J., Buta M., Rolbiecki D. 2020. The prevalence of drug-resistant and virulent *Staphylococcus* spp. in a municipal wastewater treatment plant and their spread in the environment. Environment International 143, 105914. https://doi.org/10.1016/j.envint.2020.105914. Ministry of Education and Science scoring$_{2021}$: 140; IF$_{2021}$: 13.352; PhD student participation: 55%
3. **Zieliński W**., Korzeniewska E., Harnisz M., Drzymała J., Felis E., Bajkacz S. 2021. Wastewater treatment plants as a reservoir of integrase and antibiotic resistance genes – An epidemiological threat to workers and environment. Environment International 156, 106641. https://doi.org/10.1016/j.envint.2021.106641. Ministry of Education and Science scoring$_{2021}$: 140; IF$_{2021}$: 13.352; PhD student participation: 60%
4. **Zieliński W**., Hubeny J., Buta-Hubeny M., Rolbiecki D., Harnisz M., Paukszto Ł., Korzeniewska E. 2022. Metagenomics analysis of probable transmission of determinants of antibiotic resistance from wastewater to the environment – A case study. Science of the Total Environment 827, 154354. https://doi.org/10.1016/j.scitotenv.2022.154354. Ministry of Education and Science scoring$_{2021}$: 200; IF$_{2021}$: 10.753; PhD student participation: 60%

The research presented in the publications making up the doctoral dissertation was financed from projects organized by the National Science Center No. 2017/27/B/NZ9/00267 entitled "Drug resistance of environmental microorganisms in the perspective of the holistic “One Health” approach" obtained by the supervisor Professor Ewa Korzeniewska, Dr. Habil., Eng. and No. 2017/M/NZ9/00071 entitled "Metagenomic profile of antibiotic resistance genes and genetic mobile elements in the wastewater of a municipal wastewater treatment plant" obtained by Professor Grażyna Płaza, Dr. Habil. Wiktor Zieliński is a recipient of a scholarship from the Program Interdisciplinary Doctoral Studies in Bioeconomy (POWR.03.02.00 00 I034/16 00), which is funded by the European Social Funds.



NARODOWE CENTRUM NAUKI



European Funds
Knowledge Education Development

Table of Contents

Abbreviation list: **7**

Abstract **8**

Streszczenie **14**

Introduction **21**

Scientific objective, hypotheses and scope of the study **25**

Research methods and results **26**

Stage I *26*

Stage II *27*

Stage III *32*

Stage IV *36*

Summary and Conclusions **42**

Bibliography **44**

Appendices – publications presenting the candidate's doctoral research:

Appendix 1

Zieliński W., Buta M., Hubeny J., Korzeniewska E., Harnisz M., Nowrotek M., Płaza G. 2019. Prevalence of Beta Lactamases Genes in Sewage and Sludge Treated in Mechanical-Biological Wastewater Treatment Plants. Journal of Ecological Engineering 20(9), 80–86. https://doi.org/10.12911/22998993/112506

Appendix 2

Zieliński W., Korzeniewska E., Harnisz M., Hubeny J., Buta M., Rolbiecki D. 2020. The prevalence of drug-resistant and virulent *Staphylococcus* spp. in a municipal wastewater treatment plant and their spread in the environment. Environment International 143, 105914. https://doi.org/10.1016/j.envint.2020.105914

Appendix 3

Zieliński W., Korzeniewska E., Harnisz M., Drzymała J., Felis E., Bajkacz S. 2021. Wastewater treatment plants as a reservoir of integrase and antibiotic resistance genes – An epidemiological threat to workers and environment. Environment International 156, 106641. https://doi.org/10.1016/j.envint.2021.106641

Appendix 4

Zieliński W., Hubeny J., Buta-Hubeny M., Rolbiecki D., Harnisz M., Paukszto Ł., Korzeniewska E. 2022. Metagenomics analysis of probable transmission of determinants of antibiotic resistance from wastewater to the environment – A case study. Science of the Total Environment 827, 154354

Abbreviations list:

ARB – Antibiotic Resistance Bacteria

ARG – Antibiotic Resistance Gene

AS – Activated Sludge

DRW – Downstream River Water

ECDC – European Centre for Disease Prevention and Control

ERIC – Enterobacterial Repetitive Intergenic Consensus

HGT – Horizontal Gene Transfer

HMs – Heavy Metals

IS – Insertion Sequences

MGE – Mobile Genetic Element

MLS – Macrolide-Lincosamide-Streptogramin

PBS – Phosphate-Buffered Saline

PCR – Polymerase Chain Reaction

RPK – Reads per Kilobase

qPCR – Quantitative Polymerase Chain Reaction

TPs – Transformation Products

TWW – Treated Wastewater

URT – Upper Respiratory Tract

URW – Upstream River Water

UWW – Untreated Wastewater

WHO – World Health Organization

WWTP – Wastewater Treatment Plant

Abstract

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_{TEM}, bla_{OXA}, bla_{SHV}) in the DNA isolated from the analyzed samples was determined by standard polymerase chain reaction (PCR) The bla_{TEM}, bla_{OXA} and bla_{SHV} genes were identified in all wastewater samples from the WWTP located in a forested area, in both seasons. The bla_{TEM} and bla_{OXA} genes were detected in wastewater samples from the WWTP located in an urbanized area, in both seasons. The bla_{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_{TEM} and bla_{SHV} genes were identified in river water sampled upstream from the WWTP located in a forested area, in both seasons. The bla_{OXA} gene was detected in river water sampled downstream from this WWTP in summer, whereas the bla_{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_{SHV} gene was not found in summer. In both seasons, river water sampled downstream from this WWTP contained the bla_{TEM} and bla_{OXA} genes, whereas the bla_{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.

Streszczenie

Mechaniczno-biologiczne oczyszczalnie ścieków stanowią istotne źródło bakterii antybiotykoopornych (ARB) oraz genów antybiotykooporności (ARGs), które przedostając się wraz ze ściekami odpływającymi do rzek mogą być dalej rozpowszechniane w środowisku. Wraz z bioaerozolem emitowanym podczas mieszania i napowietrzania ścieków w bioreaktorach z osadem czynnym może również dochodzić do transmisji genetycznych determinantów antybiotykooporności do górnych dróg oddechowych pracowników oczyszczalni ścieków, co stwarza potencjalne ryzyko zdrowotne oraz może wspierać rozprzestrzenianie antybiotykooporności za pośrednictwem horyzontalnego transferu genów. Celem pracy było określenie czy konwencjonalna oczyszczalnia ścieków przyczynia się do transmisji potencjalnie chorobotwórczych mikroorganizmów oraz genetycznych determinantów antybiotykooporności do środowiska wodnego oraz do górnych dróg oddechowych pracowników oczyszczalni. Badania wchodzące w skład rozprawy doktorskiej zostały podzielone na 4 etapy, w ramach których opublikowano łącznie 4 artykuły.

W pierwszym etapie badań skupiono się na analizie występowania genów warunkujących oporność na leki beta-laktamowe w wodzie rzecznej, osadzie ściekowym oraz w ściekach pozyskanych z kolejnych etapów oczyszczania ścieków pochodzących z dwóch oczyszczalni ścieków wykorzystujących technologię osadu czynnego. Próbki badawcze pobierano w dwóch sezonach badawczych (lato, jesień). W DNA wyizolowanym z próbek określono z wykorzystaniem standardowej łańcuchowej reakcji polimerazy (PCR) występowanie genów oporności na beta-laktamy (*bla*$_{\mathrm{TEM}}$, *bla*$_{\mathrm{OXA}}$, *bla*$_{\mathrm{SHV}}$). Geny *bla*$_{\mathrm{TEM}}$, *bla*$_{\mathrm{OXA}}$ oraz *bla*$_{\mathrm{SHV}}$ zidentyfikowano we wszystkich analizowanych próbkach ścieków z oczyszczalni usytuowanej na terenie zalesionym. W oczyszczalni ścieków na terenie zurbanizowanym, w obu sezonach w próbkach ścieków zidentyfikowano geny *bla*$_{\mathrm{TEM}}$ oraz *bla*$_{\mathrm{OXA}}$. Gen *bla*$_{\mathrm{SHV}}$ obecny był w ściekach pobieranych w sezonie jesiennym na wszystkich etapach oczyszczania, natomiast nie wykryto go w ściekach oczyszczonych pozyskanych w sezonie letnim. W obu sezonach w próbkach wody rzecznej przed zrzutem ścieków z oczyszczalni na terenie zalesionym zidentyfikowano geny *bla*$_{\mathrm{TEM}}$ oraz *bla*$_{\mathrm{SHV}}$. W wodzie po zrzucie ścieków w sezonie letnim zaobserwowano występowanie genu *bla*$_{\mathrm{OXA}}$ oraz nie wykryto obecności genu *bla*$_{\mathrm{SHV}}$. W sezonie jesiennym w wodzie rzecznej po zrzucie ścieków stwierdzono występowanie wszystkich analizowanych genów. W próbkach wody rzecznej przed zrzutem ścieków z oczyszczalni na terenie zurbanizowanym w sezonie jesiennym zidentyfikowano obecność wszystkich analizowanych genów, zaś w sezonie letnim nie zaobserwowano wyłącznie

obecności genu *bla*$_{SHV}$. W wodzie po zrzucie ścieków oczyszczonych w obu sezonach zidentyfikowano obecność genów *bla*$_{TEM}$ oraz *bla*$_{OXA}$, zaś nie zidentyfikowano obecności genu *bla*$_{SHV}$. Zaobserwowane zależności mogą sugerować, iż procesy oczyszczania ścieków nie usuwają całkowicie genów antybiotykooporności ze ścieków, co może stwarzać ryzyko transmisji tych genów do środowiska wodnego.

Celem badań drugiego etapu była analiza występowania antybiotykoopornych oraz wirulentnych szczepów z rodzaju *Staphylococcus* w próbkach wody rzecznej, ścieków oraz wymazów z górnych dróg oddechowych pracowników oczyszczalni ścieków. Do badań pobrano próbki wody rzecznej przed i po zrzucie ścieków, ścieki nieoczyszczone, ścieki z bioreaktora z osadem czynnym, ścieki oczyszczone, a także wymazy z nosa i gardła od pracowników oczyszczalni oraz od grupy kontrolnej. Próbki pobierane były trzy razy w ciągu roku (zima, lato, jesień). Następnie, z próbek wyizolowano genomowe DNA i wykonano identyfikację genu nukleazy oraz genów antybiotykooporności. Próbki posłużyły również do hodowli bakterii z rodzaju *Staphylococcus*, w rezultacie czego uzyskano 249 szczepów bakteryjnych, u których z wykorzystaniem PCR analizowano występowanie genu nukleazy (*nuc*), genów wirulencji oraz genów antybiotykooporności. Spośród analizowanych szczepów z rodzaju *Staphylococcus* niemalże we wszystkich analizowanych próbkach co najmniej jeden szczep posiadał gen *nuc*. Spośród wszystkich analizowanych szczepów pochodzących ze ścieków, wody rzecznej oraz wymazów od grupy kontrolnej nie zidentyfikowano genu *mec*A, który warunkuje oporność na metycylinę. Gen ten obecny był u szczepów wyizolowanych z wymazów z górnych dróg oddechowych od pracowników oczyszczalni ścieków. Gen *van*A odpowiedzialny za oporność na wankomycynę, w największym odsetku zidentyfikowano u szczepów ze ścieków z bioreaktora z osadem czynnym (49%). Spośród genów wirulencji w największej liczbie zidentyfikowano gen *hla* oraz *pvl* i *sas*X, zaś najrzadziej wykrywano *tst1* i *sec*. W kolejnej części badań w ramach tego etapu prac wykonano analizę pokrewieństwa filogenetycznego pomiędzy wszystkimi szczepami z rodzaju *Staphylococcus*. Wykonano dendrogramy na podstawie wzorców elektroforetycznych ERIC-PCR i rozpowszechnienia analizowanych genów, co miało na celu wykazać pokrewieństwo między szczepami ze ścieków oczyszczonych oraz wody rzecznej po zrzucie ścieków oraz między szczepami ze ścieków i wymazami od pracowników oczyszczalni ścieków. Zaobserwowano wysokie podobieństwo genetyczne pomiędzy szczepami ze ścieków z bioreaktora z osadem czynnym i szczepami izolowanymi od pracowników. Wyniki te mogą sugerować występowanie potencjalnej transmisji tych mikroorganizmów wraz z bioaerozolem emitowanym podczas napowietrzania i

mieszania ścieków w bioreaktorach z osadem czynnym do górnych dróg oddechowych pracowników oczyszczalni ścieków. W ostatniej części badań w ramach tego etapu prac, w celu zbadania koncentracji genów *nuc*, *mec*A, *van*A and *qac*A/B wykonano analizę qPCR w DNA środowiskowym próbek. Gen *nuc* w największej koncentracji wykryto w ściekach nieoczyszczonych (7.76×10^3 to 5.87×10^4 gene copies/mL) oraz w ściekach z bioreaktora z osadem czynnym (2.72×10^3 to 1.82×10^4 gene copies/mL). Gen *mec*A zidentyfikowano w próbkach ścieków nieoczyszczonych w każdym z badanych sezonów w zakresie koncentracji od 3.05×10^2 do 2.68×10^3 gene copies/mL. Gen *van*A zidentyfikowano w próbkach ścieków nieoczyszczonych we wszystkich sezonach badawczych (średnio 8.28×10^5 gene copies/mL), zaś *qac*A/B został wykryty we wszystkich próbkach z wyjątkiem próbek ścieków z bioreaktora z osadem czynnym (1.02×10^2 to 6.97×10^5 gene copies/mL). Wyniki badań wykazują, że oczyszczalnie ścieków są źródłem genów antybiotykooporności, zaś ścieki oczyszczone odpływające do rzeki mogą wpływać na ich rozprzestrzenianie w środowisku.

Badania przeprowadzone w etapie trzecim miały na celu określenie stopnia potencjalnej transmisji wybranych zanieczyszczeń wraz ze ściekami oczyszczonymi do wody rzecznej oraz wraz z bioaerozolem emitowanym podczas napowietrzania i mieszania ścieków do górnych dróg oddechowych pracowników oczyszczalni ścieków. Analizie poddano próbki wody rzecznej przed i po zrzucie ścieków, ścieki nieoczyszczone, ścieki z bioreaktora z osadem czynnym, ścieki oczyszczone, a także wymazy z nosa i gardła od pracowników oczyszczalni oraz od grupy kontrolnej. Próbki pobierane były trzykrotnie w ciągu roku (zima, lato, jesień) ze względu na potencjalne różnice w spożyciu antybiotyków w różnych sezonach. W uzyskanym materiale genetycznym identyfikowano geny warunkujące antybiotykooporność, geny oporności na metale ciężkie oraz geny integrazy z wykorzystaniem metody qPCR. Ponadto, w próbkach zbadano koncentrację wybranych parametrów fizykochemicznych, metali ciężkich oraz antybiotyków popularnie stosowanych w lecznictwie. Wszystkie z 17 analizowanych ARGs wykryto w co najmniej jednej próbce ścieków lub wody rzecznej. W próbkach ścieków najwyższe stężenia osiągnęły geny odpowiedzialne za oporność na beta-laktamy oraz antybiotyki z grupy MLS. Zaobserwowano spadek koncentracji większości ARGs podczas kolejnych etapów oczyszczania ścieków. W próbkach wody rzecznej wykryto 13 spośród 17 badanych ARGs oraz zaobserwowano, że wszystkie ARGs w próbkach DRW występowały w stężeniach wyższych w porównaniu do ich stężeń występujących w URW. Spośród genów integrazy dominował gen *int*I1, natomiast w przypadku genów oporności na metale ciężkie zidentyfikowano wyłącznie gen *cnr*A. Kolejną częścią badań w ramach tego

etapu było określenie relatywnych koncentracji (gene copies/16S rRNA copies) wybranych pięciu genów antybiotykooporności – *bla*$_{TEM}$, *Amp*C, *sul*1, *erm*F, *mef*A oraz genu integrazy klasy pierwszej – *int*I1 w DNA wyizolowanym z próbek wymazów od pracowników oczyszczalni ścieków oraz od osób z grupy kontrolnej nie związanych z WWTP. Stwierdzono, że większość analizowanych genów występuje w wyższych stężeniach w DNA z wymazów pobranych od personelu oczyszczalni niż od grupy kontrolnej. W próbkach wymazów z gardła dominowały geny *erm*F (2.62×10^{-2}–7.45×10^{-3} gene copies/16S rRNA copies) oraz *mef*A (1.54×10^{-1}–4.84×10^{-2} gene copies/16S rRNA copies), zaś w próbkach z wymazów z nosa dominowały geny *int*I1 (5.81–7.72×10^{-1} gene copies/16S *r*RNA copies) oraz *sul*1 (1.74–2.62×10^{-2} gene copies/16S rRNA copies). Wszystkie badane geny obecne w DNA z próbkach wymazów od pracowników oczyszczalni zidentyfikowano również w DNA pochodzącym ze ścieków z bioreaktora z osadem czynnym. Pracownicy mechaniczno-biologicznych oczyszczalni ścieków, w których występuje intensywna emisja bioaerozoli, są narażeni na ryzyko przeniesienia do górnych dróg oddechowych ARGs ze ścieków. Analiza współczynnika korelacji Spearman'a wykazała wiele statystycznie istotnych pozytywnych korelacji między koncentracją wybranych parametrów fizycznych, a stężeniem analizowanych ARGs. Na podstawie uzyskanych wyników stwierdzono, że środowiskowe parametry fizykochemiczne mogą mieć wpływ na zmiany obecności i liczby ARGs w zbiornikach wodnych oraz na ich transmisję w środowisku. Istotne znaczenie ma głównie obecność pozostałości antybiotyków i produktów ich rozpadu (TPs). Stwierdzono, że próbki ścieków nieoczyszczonych i oczyszczonych zawierają 13 z 16 analizowanych antybiotyków, zaś najwyższymi stężeniami w próbkach ścieków nieoczyszczonych charakteryzowały się: trimetoprim (2.45–1643.74 ng/L), cyprofloksacyna (92.16–306.79 ng/L), klarytromycyna (69.30–401.79 ng/L), sulfametoksazol (118.82–160.27 ng/L) oraz doksycyklina (16.44–97.91 ng/L). Zaobserwowano, że próbki ścieków oczyszczonych zawierały wszystkie antybiotyki zidentyfikowane w ściekach nieoczyszczonych. W wodzie rzecznej poniżej punktu zrzutu ścieków stężenia większości analizowanych antybiotyków były wyższe niż w próbkach przed ich zrzutem. Skuteczność usuwania leków w procesie technologicznym była zróżnicowana w zależności od rodzaju antybiotyku i pory roku. W sezonie zimowym oraz letnim zaobserwowano całkowite usunięcie lewofloksacyny oraz wysoki stopień usunięcia sulfadiazyny (87.7–94%), sulfametoksazolu (81.8–84%) i cyprofloksacyny (79.6–82.6%). W próbkach ścieków nieoczyszczonych stężenia wszystkich metali ciężkich mieściły się w granicach dopuszczalnych przez obowiązujące w Polsce wytyczne (MEIN 2019), natomiast w ściekach z bioreaktora z osadem czynnym zawartość cynku (1.47–2.39 mg/L) w każdej z próbek przekroczyła wartości progowe (≤1

mg/L). Procesy oczyszczania ścieków stosowane w układzie technologicznym oczyszczalni doprowadziły do dużego spadku zawartości HMs w ściekach oczyszczonych, co może być wynikiem ich kumulacji w osadach ściekowych usuwanych z oczyszczalni. W badaniach zaobserwowano statystycznie istotne pozytywne korelacje między koncentracjami genów integrazy trzech klas, a szeroką gamą ARGs, pomiędzy koncentracjami genu *cnr*A oraz 12 ARGs, a także między koncentracjami genów integrazy i stężeniem antybiotyków. Wykazano również pozytywne istotne statystycznie korelacje (r = 0.60–0.96) między koncentracją ARGs, a stężeniem metali ciężkich, spośród których wyróżniały się zawartość cynku oraz miedzi. Wyniki potwierdzają ważną rolę integronów w rozpowszechnianiu antybiotykooporności.

Celem przeprowadzonych badań w etapie czwartym było określenie: 1) czy w oczyszczalni ścieków wykorzystującej technologię osadu czynnego dochodzi do transmisji czynników antybiotykooporności wraz z bioaerozolem wytwarzanym podczas oczyszczania ścieków do górnych dróg oddechowych pracowników oczyszczalni, a także 2) czy wraz ze ściekami oczyszczonymi odpływającymi do rzeki mogą one przedostawać się do zbiorników wód powierzchniowych. Do badań pobrano próbki wody rzecznej, ścieków oraz wymazów od pracowników oczyszczalni ścieków, które poddano głębokiemu sekwencjonowaniu nowej generacji. W próbkach określano zawartość genów antybiotykooporności oraz mobilnych elementów genetycznych, a także skład taksonomiczny mikroorganizmów. Największe zróżnicowanie taksonomiczne zaobserwowano w próbkach ścieków nieoczyszczonych, a najmniejsze w próbkach ścieków oczyszczonych. Dominującymi gromadami bakteryjnymi były Proteobacteria, Firmicutes, Actinobacteria oraz Bacteroidetes. Próbki URW charakteryzowały się niższą bioróżnorodnością w porównaniu do próbek DRW. W wymazach od pracowników zaobserwowano podobny skład taksonomiczny mikroorganizmów, dominantami były głównie Firmicutes i Actinobacteria i Bacteroidetes. Względna obfitość ARGs wahała się od 2.88×10^{-5} kopii genów/16S rRNA do 1.27 kopii genów/16S rRNA w próbkach z UWW; od 1.3×10^{-4} kopii genów/16S rRNA kopii do 9.0×10^{-2} kopii genów/16S rRNA kopii w próbkach AS; i od 2.62×10^{-5} kopii genu/16S rRNA kopii do 7.3×10^{-1} kopii genu/16S rRNA kopii w próbkach TWW. Dominującymi typami ARGs były geny oporności wielolekowej, a następnie geny kodujące oporność na leki z grupy MLS, beta-laktamy oraz tetracykliny. Liczebność ARGs w próbkach wody wahała się od 1.84×10^{-5} kopii genów/16S rRNA do 2.244 kopii genów/16S rRNA kopii w próbkach URW oraz od 9.98×10^{-6} do 1.83 kopii genów/16S rRNA kopii w próbkach DRW. W próbkach wody najbardziej licznie występowały geny oporności wielolekowej, geny kodujące oporność na beta-laktamy, MLS

oraz aminoglikozydy. Zaobserwowano większą różnorodność ARGs w próbach DRW w porównaniu do URW. Koncentracja ARGs w wymazach z nosa wahała się od 3.96×10^{-5} gene copies/16S rRNA copies do 4.61×10^{-1} gene copies/16S rRNA copies, natomiast w wymazach z gardła od 1.38×10^{-5} gene copies/16S rRNA copies do 1.57×10^{-1} gene copies/16S rRNA. W wymazach z nosa dominowały geny oporności na leki MLS, aminoglikozydy oraz chloramfenikol, natomiast w gardle – geny oporności na beta-laktamy, tetracykliny i antybiotyki z grupy MLS. Zidentyfikowane rodziny sekwencji insercyjnych występowały w największej liczbie w ściekach nieoczyszczonych, a do najliczniej występujących zaliczono IS3, IS4 i IS21. W próbkach wody po zrzucie zaobserwowano większą liczebność IS niż w wodzie przed zrzutem ścieków, a najbardziej rozpowszechnionymi były IS3, IS4 oraz IS5. W wymazach z nosa i gardła zaobserwowano zbliżone wartości IS, dominowały zaś IS3, ISL3 i IS30. Analiza ARGs w kontekście transmisji determinantów antybiotykooporności oraz mikroorganizmów wraz ze ściekami do wody rzecznej wykazała, że względna liczebność prawie wszystkich badanych ARGs była wyższa w próbkach DRW w porównaniu do ich ilości w próbkach URW. Ponadto próbki DRW zawierały ARGs, które nie zostały wykryte w próbkach URW. W próbkach URW i DRW najliczniejszymi ARGs były *bac*A, *bcr*A, *emr*E, *mex*D, *aph(3")-I*, *sul*1, *tet*Q oraz *erm*F. Zaobserwowano również, że 8 z 10 analizowanych rodzin IS występowało liczniej w próbkach DRW w porównaniu z URW. Analiza współczynnika korelacji, wykazała, że zidentyfikowane gromady bakterii skorelowane są ze stężeniami analizowanych ARGs oraz sekwencjami insercyjnymi. Analiza danych w kontekście transmisji mikroorganizmów oraz determinantów antybiotykooporności do górnych dróg oddechowych pracowników oczyszczalni ścieków wykazała, że część z ARGs zidentyfikowano w wymazach z nosogardzieli w koncentracji zbliżonej do ich koncentracji w próbkach ścieków. W wymazach z nosa dominowały *erm*C, *erm*X, *ant(2")-I*, *sul*1, natomiast w gardle przewyższały *cfx*A2, *tet*M, *tet*Q oraz *erm*B. Zaobserwowano pozytywne istotne statystycznie korelacje między koncentracjami ARGs, a liczebnością gromad mikroorganizmów, które zidentyfikowano zarówno w wymazach od pracowników oraz w ściekach. W próbkach wymazów od pracowników oczyszczalni zidentyfikowano sekwencje insercyjne, które nie są charakterystyczne dla ludzkiego mikrobiomu, a zostały wykryte w próbkach ścieków nieoczyszczonych i z bioreaktora z osadem czynnym. Wyniki wskazują, że odprowadzanie ścieków zwiększa pulę genetycznych determinantów antybiotykooporności w wodach powierzchniowych, a także wskazują na niebezpieczeństwo ich transmisji do górnych dróg oddechowych pracowników oczyszczalni ścieków. Przeprowadzone badania wskazują, że wraz z emisją bioaerozoli podczas oczyszczania ścieków może dochodzić do transmisji

antybiotykoopornych mikroorganizmów oraz determinantów antybiotykooporności do górnych dróg oddechowych pracowników oczyszczalni ścieków, co może stanowić zagrożenie zdrowotne i epidemiologiczne. Badania podkreślają potrzebę wdrożenia środków ochronnych i zapobiegawczych dla pracowników oczyszczalni ścieków, a także opracowania i wdrożenia nowych technologii oczyszczania ścieków w celu zminimalizowania emisji bioaerozolu. Ponadto, badania wykazały, że wraz ze ściekami oczyszczonymi odpływającymi z oczyszczalni ścieków może dochodzić do odprowadzania do rzeki szerokiej gamy antybiotykoopornych oraz potencjalnie chorobotwórczych mikroorganizmów, determinantów antybiotykooporności oraz zanieczyszczeń chemicznych.

Introduction

Bacterial antimicrobial resistance poses one of the greatest challenges for modern medicine and constitutes a serious threat for public health. In recent decades, this problem has been significantly exacerbated by the widespread use of antimicrobials in human and veterinary medicine and in agriculture. The emergence of new drug resistance mechanisms in bacteria and the spread of antibiotic resistance is a worrying phenomenon that poses a serious threat to public health by increasing medical costs, decreasing treatment efficacy, prolonging hospital stays, and increasing mortality. The number of antibiotic-resistant bacteria (ARB) continues to increase around the world (Pazda et al., 2019). In the countries of the European Union (EU) and the European Economic Area (EEA), ARB caused around 670,000 infections and accounted for more than 33,000 attributable deaths (Cassini et al., 2019). According to the World Health Organization (WHO, 2014), antimicrobial resistance poses a serious threat for human and animal health, food security and global economic growth. As postulated by the One Health multidisciplinary approach, these threats should be urgently addressed to avoid a public health crisis in the coming years.

Antibiotics play a fundamental role in the treatment of bacterial infections. The global spread of antimicrobial resistance decreases the number of effective antibiotic treatments. Overuse and unjustified use of antimicrobials, including last-resort antibiotics that should be prescribed to treat specific infections, as well as high doses of antibiotics applied in the clinical setting, significantly contribute to the spread of antimicrobial resistance (Karam et al., 2016; Munita and Arias, 2016). According to the European Center for Disease Prevention and Control (ECDC), the consumption of antimicrobials in the EU and EEA has remained high for several decades. In 2019, total antimicrobial consumption (community and hospital sector combined) in the EU/EEA reached 19.4 defined daily doses (DDD) per 1000 inhabitants. In Poland, this parameter reached 23.6 DDD, exceeding the EU/EEA average. Penicillins were the most frequently used antimicrobials in nearly all European countries (including Poland), and they accounted for 27% (Slovakia) to 66% (Denmark) of total antibiotic consumption in Europe (ECDC, 2020). Antibiotics and their transformation products (TPs) are not fully re moved from wastewater in wastewater treatment plants, and these pollutants are released to the environment with effluents (Guo et al., 2018; Rizzo et al., 2013).

Long-term exposure to antibiotics leads to the proliferation and spread of ARB and antibiotic resistance genes (ARGs) in various ecosystems (Lopatkin et al., 2016). Both ARB and ARGs are ubiquitous in the environment, and human activities significantly contribute to

their growth and transmission. Wastewater treatment plants (WWTPs) are regarded as reservoirs of antibiotic-resistant microorganisms and hotspots for the transmission of ARGs and ARB in the environment (Roberto et al., 2019). Wastewater is highly abundant in microorganisms, carbon and other nutrients, and the physicochemical conditions inside WWTPs create a highly supportive environment for microbial growth (Da Silva et al., 2006; Grehs et al., 2021; Maraschin et al., 2020; Silveira et al., 2020). Chemical micropollutants, including residual antibiotics, are not completely removed from treated wastewater (Beretsou et al., 2020), which contributes to the growth and proliferation of resistant microorganisms due to selective pressure. In WWTPs, organic and inorganic pollutants are removed from wastewater, and research has demonstrated that ARG concentrations and ARB counts can be reduced during treatment (Osińska et al., 2020). Despite the above, there is considerable evidence to indicate that large amounts of these micropollutants are evacuated to the environment with effluents (Jia et al., 2018; Lekunberri et al., 2018; Proia et al., 2016). When treated wastewater is discharged to surface water bodies, ARGs and ARB spread in the environment, which can lead to ecological stress and disrupt the balance in natural ecosystems. These observations indicate that WWTPs significantly contribute to the transmission of ARGs and ARB in the environment, thus compromising the safety of surface water and posing a potential threat for public health. In WWTPs with a mechanical biological treatment system, additional physical and/or chemical treatment methods should be introduced to effectively remove these pollutants. According the literature, ARG concentrations in wastewater can be reduced by applying high-intensity UV disinfection lamps (Guo et al., 2013) and advanced oxidation processes such as Fenton's reaction, photocatalysis with TiO_2 and UV/H_2O_2 (Felis et al., 2022; Rizzo et al., 2014), and coagulation (Li et al., 2017).

Microorganisms, in particular bacteria colonizing environments contaminated with antibiotics, drug residues and other pollutants, can rapidly adapt to unsupportive conditions through a mechanism known as horizontal gene transfer (HGT) (Felden and Cattoir, 2018). High counts of microorganisms that carry diverse mobile genetic elements (MGEs) contribute to HGT. These elements promote antimicrobial resistance by capturing, storing and disseminating resistance determinants both within (intracellular mobility) and between (intercellular mobility) microorganisms (Partridge et al., 2018). Mobile genetic elements such as integrons are one of the key contributors to the spread of antibiotic resistance in environmental bacterial strains (Ghaly et al., 2019). Long-term research has demonstrated that integrons are present in various environments, including water bodies, wastewater and soil

(Buta et al., 2021). The integrase gene plays a key role in integron activity, and it catalyzes the insertion of exogenous gene cassettes into integrons. A gene cassette can contain a single gene or a group of genes with diverse functions (Domínguez et al., 2019).

Heavy metals (HMs) are the most toxic pollutants found in wastewater. Heavy metals do not undergo biodegradation, and they tend to accumulate in soil and sewage sludge (Gogoi et al., 2018; Tytła, 2019). Although HMs are naturally occurring elements, anthropogenic activities such as chemical production, fuel combustion, and modern agricultural practices (Dickinson et al., 2019) are responsible for growing levels of HM pollution in the environment. High concentrations of HMs in the environment promote the maintenance and spread of antibiotic resistance via three potential co-selection mechanisms (Mazhar et al., 2021), namely co-occurrence, co-resistance, and co-regulation. Co-occurrence takes place when genes encoding resistance to both HMs and antibiotics are grouped on the same MGE. Co-resistance is a mechanism where antibiotics and HMs initiate the same biochemical paths and lead to the emergence of HM-induced antibiotic resistance (Imran et al., 2019). Co-regulation is a process where a single regulatory gene controls the expression of genes that confer resistance to both HMs and antibiotics (Rensing et al., 2018).

Air pollution is rising at an alarming rate around the globe. Air pollutants include biological aerosols (bioaerosols) which are composed of microorganisms and their metabolites, microbial toxins, and other liquid and solid particles suspended in the air (Han et al., 2013). Conventional mechanical and biological WWTPs are regarded as a source of bioaerosol emissions. Massive amounts of bioaerosols are released into the air during preliminary mechanical treatment, aeration and mixing of wastewater in activated sludge bioreactors. Inhaled bioaerosols pose a health threat for WWTP plant employees and local residents (Byliński et al., 2019). Bioaerosol emissions are affected by various factors, such as the plant's geographic location, weather, composition and origin of wastewater, treatment technology, and the plant's capacity (Han et al., 2018). Depending on its origin, wastewater can contain numerous pathogens, including bacteria of the genera *Bacillus*, *Clostridium*, *Escherichia*, *Klebsiella*, *Mycobacterium*, *Enterococcus*, *Kocuria*, *Micrococcus*, *Staphylococcus*, and *Streptomyces* (Maki et al., 2017; Yang et al., 2019). According to the literature, the most common health problems reported by WWTP employees include gastrointestinal disorders, fever, headache, vertigo, fatigue, difficulty breathing, skin rash, and eye, nose and throat irritation (Uhrbrand et al., 2011). These problems can also affect local residents because bioaerosols can be carried in the air stream over a distance of up to 10 km from the WWTP (Li

et al., 2016). The bioaerosols transmitted to the upper respiratory tracts of WWTP employees contain microbial gene pools, including genes that encode resistance to antimicrobials and other pharmaceuticals, as well as MGEs. Previous research has shown that bioaerosols emitted in WWTPs contain antimicrobial resistance determinants which can pose an epidemiological risk because bacteria can acquire ARGs through HGT (Gaviria-Figueroa et al., 2019; Korzeniewska et al., 2013; Korzeniewska and Harnisz, 2013). To minimize these threats, effective technologies should be developed to limit bioaerosol emissions in WWTP, adequate safety procedures should be introduced, and plant employees should be provided with personal protective equipment. There is scant research on the health risks associated with bioaerosol exposure, which is why WWTPs should be regularly monitored for the presence of ARB and ARG and their potential transmission.

Scientific objective, hypotheses and scope of the study

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.

Research hypotheses

1. Wastewater treated in WWTPs that rely on a mechanical biological treatment system and the activated sludge technology increases the abundance of antibiotic-resistant microorganisms and the genetic determinants of antibiotic resistance in water bodies receiving effluents.
2. In WWTPs that rely on a mechanical biological treatment system and the activated sludge technology, antibiotic-resistant microorganisms and the genetic determinants of antibiotic resistance are transmitted, with the bioaerosol emitted during wastewater mixing and aeration, to the upper respiratory tracts of WWTP employees, which poses potential epidemiological and health threats.

Scope of the study

1. To determine the prevalence of the genetic determinants of antibiotic resistance and antibiotic-resistant microorganisms in wastewater in each stage of treatment in WWTPs that rely on a mechanical biological treatment system and the activated sludge technology (publications 1, 2, 3, 4)
2. To determine whether the genetic determinants of antibiotic resistance and antibiotic-resistant microorganisms are transmitted with treated wastewater to the aquatic environment (publications 2, 3, 4)
3. To determine the prevalence of chemical contaminants (antibiotics and HMs) in wastewater and to analyze whether they can reach surface water bodies with effluents (publication 3)
4. To determine whether the genetic determinants of antibiotic resistance and antibiotic-resistant microorganisms can be transmitted from wastewater to the upper respiratory tracts of employees in WWTPs that rely on a mechanical biological treatment system and the activated sludge technology (publications 2, 3, 4)

Research methods and results

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. Each stage of the study was designed so as to determine the prevalence of the genetic determinants of antibiotic resistance and antibiotic-resistant microorganisms in wastewater and their transmission to (1) rivers receiving effluents and (2) the upper respiratory tracts of WWTP employees with the bioaerosol emitted during the mechanical and biological stages of wastewater treatment.

Stage I

Aim: 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.

Materials and Methods: Samples of river water collected upstream and downstream from the wastewater discharge point, sewage sludge and wastewater in successive treatment stages in two WWTPs relying on the activated sludge technology, located in two different regions of Poland: (1) a forested non-industrialized area and (2) an urbanized and industrialized area, were analyzed in the study. The samples were collected in two seasons (summer, fall). Wastewater and river water samples were passed through 0.2 μm filters, and genomic DNA was isolated with the DNeasy Power Water Kit (Qiagen). DNA was also extracted from sewage sludge with the DNeasy Power Soil Kit (Qiagen). The presence of beta-lactamase genes (*bla*$_{TEM}$, *bla*$_{OXA}$, *bla*$_{SHV}$) in the genomic DNA isolated from the analyzed samples was determined by PCR.

Results:

Samples of river water collected upstream and downstream from the wastewater discharge point, sewage sludge and wastewater collected in each stage of the treatment process were analyzed to determine the presence of three genes encoding resistance to beta-lactam antibiotics: *bla*$_{TEM}$, *bla*$_{OXA}$ and *bla*$_{SHV}$. Differences in the prevalence of the analyzed genes in samples were found between the two WWTPs and sampling seasons. The *bla*$_{TEM}$, *bla*$_{OXA}$ and *bla*$_{SHV}$ genes were identified in all wastewater samples from the WWTP located in a forested area, in both seasons. The *bla*$_{TEM}$ and *bla*$_{OXA}$ genes were detected in wastewater samples from the WWTP located in an urbanized area, in both seasons. The *bla*$_{SHV}$ 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, and it was not identified in any of the samples collected in summer.

The *bla*$_{TEM}$ and *bla*$_{SHV}$ genes were identified in river water sampled upstream from the WWTP located in a forested area, in both seasons. The *bla*$_{OXA}$ gene was detected in river water sampled downstream from this WWTP in summer, whereas the *bla*$_{SHV}$ gene was not found, which suggests that it was effectively removed during the wastewater treatment process. All analyzed genes, including *bla*$_{OXA}$ that was absent in the samples collected upstream from the WWTP, were identified in river water sampled downstream from the WWTP in fall. This observation suggests that *bla*$_{OXA}$ could potentially be transmitted with effluents to river water in both summer and 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*$_{SHV}$ gene was not found in summer. In both seasons, river water sampled downstream from this WWTP contained the *bla*$_{TEM}$ and *bla*$_{OXA}$ genes, whereas the *bla*$_{SHV}$ gene was not present. The above findings indicate that ARGs are not completely removed during the wastewater treatment process, which poses a potential risk of their transmission to the aquatic environment.

The *bla*$_{TEM}$ and *bla*$_{OXA}$ genes were identified in all sewage sludge samples (after mechanical and gravity thickening) and in leachate samples in the WWTP located in an urbanized area, in both seasons, whereas the *bla*$_{SHV}$ gene was not detected. In the WWTP situated in a forested area, the prevalence of the analyzed genes in sewage sludge samples varied across the sampling seasons. All analyzed genes were present in sludge samples collected after passage through the fermentation pool in summer, whereas the *bla*$_{SHV}$ gene was not found in fall. All examined genes were identified in sludge samples prepared for agricultural utilization in fall, whereas the *bla*$_{OXA}$ gene was not detected in summer. The research results confirmed the presence of genes encoding resistance to beta-lactam antibiotics in sludge samples. This gives cause for concern because sewage sludge is often used in industry and agriculture, which may pose a risk of their spread to other environments.

Publication:

Zieliński W., Buta M., Hubeny J., Korzeniewska E., Harnisz M., Nowrotek M., Płaza G. 2019. Prevalence of Beta Lactamases Genes in Sewage and Sludge Treated in Mechanical-Biological Wastewater Treatment Plants. Journal of Ecological Engineering 20(9), 80–86. https://doi.org/10.12911/22998993/112506

Stage II

Aim: The aim of the second stage of the study was to analyze the prevalence of drug-resistant and virulent *Staphylococcus* strains in river water samples, wastewater samples and upper

respiratory tract specimens collected from WWTP employees. A phylogenetic analysis was performed to determine whether bacteria of the genus *Staphylococcus* are transmitted from effluents to water, and from wastewater from an activated sludge bioreactor to the upper respiratory tracts of WWTP employees.

Materials and Methods: The study was conducted in a WWTPs that relies on a mechanical biological treatment system and the activated sludge technology. The plant receives municipal sewage and a low percentage of hospital wastewater (2%). 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 in three different seasons (winter, summer, fall) to account for potential differences in antibiotic consumption throughout the year. A total of 15 liquid samples (three samples from each sampling site) and six pooled swabs (three nasal swabs and three throat swabs) collected from 15 WWTP employees were analyzed. In addition, nasal and throat swabs were collected from the control group, i.e. healthy individuals who were not employed at the WWTP. The samples were used to culture *Staphylococcus* bacteria on the Giolitti-Cantoni liquid medium (Sigma-Aldrich, Merck) and the Baird-Parker solid medium (Sigma-Aldrich, Merck). Genomic DNA was isolated from 455 bacterial strains using the AX Gravity kit (A&A Biotechnology). In the next step, the bacterial strains were analyzed for the presence of the *rpo*B gene, characteristic of the genus *Staphylococcus*. A clonal analysis by ERIC PCR was performed to exclude identical *Staphylococcus* strains, isolated from the same samples, from further analyses. As a result, a total of 249 bacterial strains were obtained, which were analyzed for the presence of the *nuc* gene, virulence genes (*sas*X, *pvl*, *tst1*, *hla*, *sec*), the antiseptic resistance gene (*qac*A/B) and ARGs (*mec*A, *van*A). Simultaneously, genomic DNA was isolated from water, wastewater and swabs using the FastDNA Spin Kit for Feces (MB Biomedicals), and qPCR was carried out to determine the presence and abundance of the *nuc* gene (encoding nuclease activity in *S. aureus*) and genes encoding antimicrobial resistance and virulence.

Results:

In nearly all analyzed samples, at least one *Staphylococcus* strain harbored the *nuc* gene, characteristic of *S. aureus*. In untreated wastewater, the *nuc* gene was detected in as many as 96% (25 out of 26) of the examined strains; in wastewater from an activated sludge bioreactor, the *nuc* gene was identified in 86% (42 out of 59) of the strains; in treated wastewater, this gene

was found in 56% (14 out of 25) of the strains; in river water collected upstream from the wastewater discharge point, the *nuc* gene was identified in 31% of the strains. Interestingly, this gene was not detected in strains from river water collected downstream from the wastewater discharge point, although it was found in more than half of the isolates obtained from effluents. As regards upper respiratory tract specimens, the *nuc* gene was identified in 39 out of 49 nasal isolates (80%) and in 33 out of 39 throat isolates (85%). An analysis of control swabs revealed that all identified strains harbored the *nuc* gene. These findings may indicate that irrespective of contact with wastewater, *S. aureus* colonizes nasal and throat mucosa in humans.

In the next step of this stage of the study, the strains were analyzed for the presence of ARGs – the methicillin resistance gene (*mec*A) and the vancomycin resistance gene (*van*A). The *mec*A gene was not identified in any of the analyzed strains obtained from wastewater, river water or control group swabs. This gene was detected in the strains isolated from upper respiratory tract specimens collected from WWTP employees – in eight out of 49 strains (16%) from nasal swabs and in six out of 39 strains (15%) from throat swabs. The absence of *mec*A in wastewater samples may suggest that WWTP employees, similarly to control group members, could be carriers of this gene regardless of daily contact with wastewater. Interestingly, none of the strains harboring *mec*A belonged to the *S. aureus* species. The *van*A gene was identified in only one out of 26 strains (4%) obtained from untreated wastewater, whereas it was detected in nearly half of the isolates (24 out of 49) from wastewater from an activated sludge bioreactor. The *van*A gene was found in only 8% of the strains from treated wastewater. The *van*A gene was not identified in the strains isolated from river water samples collected upstream and downstream from the wastewater discharge point. As regards *Staphylococcus* isolates from swabs collected from WWTP employees, *van*A was identified in only 4% of nasal strains (two out of 49 isolates) and in 26% of throat strains (10 out of 39 isolates). The reverse relationship was observed in the strains isolated from control group swabs – none of the strains isolated from throat swabs harbored the *van*A gene, which was identified in 25% of nasal strains (three out of 12 isolates). It was also found that 77% of the strains identified as *S. aureus* based on the presence of the *nuc* gene harbored also the *van*A gene. In addition, 8% of all strains carrying the *van*A gene harbored also the *mec*A gene.

Subsequent analyses were performed to identify the chlorhexidine gluconate resistance gene (*qac*A/B) - chlorhexidine gluconate is a popular antiseptic widely used in medical facilities and virulence genes (*pvl, hla, tst1*, *sec, sas*X) in the genus *Staphylococcus.* The *qac*A/B gene was detected in 39 out of 135 (29%) *Staphylococcus* strains isolated from wastewater and river water

samples, and in 22 out of 88 (25%) isolates from upper respiratory tract specimens collected from WWTP employees; 84% of strains harboring the *qac*A/B gene were identified as *S. aureus*. In wastewater samples, the prevalence of *qac*A/B was lowest (12%) in strains isolated from an activated sludge bioreactor, higher in treated wastewater (36%), and highest in untreated wastewater (65%). In isolates obtained from river water sampled upstream and downstream from the wastewater discharge point, *qac*A/B was identified in 25% and 16% of the strains, respectively. In isolates obtained from nasal and throat swabs collected from WWTP employees, *qac*A/B was detected in 20% and 31% of the strains, respectively. In *Staphylococcus* strains, the *pvl* gene encodes a toxin that induces pore formation in leukocyte cell membrane complement receptors. The *pvl* gene was most frequently identified in strains isolated from wastewater samples from an activated sludge bioreactor (63%), and it was least frequently encountered in strains isolated from effluents (8%). This gene was detected in 49% and 39% of the strains isolated from the nasal and throat swabs of WWTP employees, respectively, whereas it was not found in control group swabs. As many as 88% of *pvl*-positive strains were identified as *S. aureus*. The *hla* gene encodes alpha-hemolysin, a pore-forming toxin which is one of the most important virulence factors associated with *S. aureus*. In the present study, 84% (122 out of 145) *hla*-positive strains were classified as *S. aureus*. The prevalence of the *hla* gene was highest in strains isolated from untreated wastewater (88%) and wastewater from an activated sludge bioreactor (73%), and it was reduced to 44% in isolates from treated wastewater. This gene was also identified in isolates from river water sampled upstream and downstream from the wastewater discharge point (38% and 21%, respectively). The *hla* gene was detected in around 75% of strains isolated from swab samples. Toxic shock syndrome is a life-threatening condition that is most frequently caused by the toxic shock syndrome toxin 1 (TSST-1) superantigen encoded by the *tst*1 gene. Another dangerous toxin produced by bacteria of the genus *Staphylococcus* is type C enterotoxin encoded by the *sec* gene. In the current study, *tst*1 and *sec* were detected least frequently, in only 2% and 5% of the isolates from wastewater and river water samples, respectively, and in 31% and 26% of the isolates from swab samples, respectively. The *sas*X gene belongs to the family of surface-located proteins that are attached to the bacterial cell, accelerate biofilm formation, cell aggregation and cell adhesion. The prevalence of *sas*X was similar in strains isolated from URW (38%), UWW (35%), AS (33%), TWW (44%) and DRW (37%) samples.

In the next step of this stage of the study, a phylogenetic analysis of all *Staphylococcus* strains was performed. Dendrograms were developed based on ERIC-PCR profiles and the prevalence

of the analyzed genes in the examined strains, separately for the isolates obtained from samples collected in different seasons. This analysis was performed to determine the relatedness between the strains isolated from treated wastewater and river water sampled downstream from the wastewater discharge point, and between the strains isolated from wastewater and swabs collected from WWTP employees. In winter, the dendrogram was divided into three clusters, one of which comprised the strains isolated from nasal swabs collected from WWTP employees, the strains isolated from throat swabs collected from WWTP employees, the strains isolated from wastewater from an activated sludge bioreactor, and treated wastewater. Relatively high genetic similarity was observed between isolates from wastewater from an activated sludge bioreactor and the strains isolated from WWTP employees (nearly all isolates were identified as *S. aureus,* and all isolates harbored the *pvl* and *hla* genes). In summer, the dendrogram was divided into four clusters, one of which consisted of the strains isolated from treated wastewater and river water sampled downstream from the wastewater discharge point. Two clusters comprised the strains isolated from WWTP employees, and the strains isolated from untreated wastewater and wastewater from an activated sludge bioreactor. The above two clusters were characterized by high genetic similarity, and all strains were identified as *S. aureus*. In fall, the dendrogram was divided into five clusters, none of which comprised the strains isolated from river water sampled upstream and downstream from the wastewater discharge point. No significant relatedness in terms of pollutant transmission was observed between four clusters, whereas the fifth cluster consisted of the strains isolated from both WWTP employees and wastewater. These strains were classified as *S. aureus,* and most of them harbored virulence genes *hla* and *sas*X.

In the last step of this stage of the study, a qPCR assay of genomic DNA from wastewater and river water samples was performed to determine the concentrations of *nuc*, *mec*A, *van*A and *qac*A/B genes. Differences in the concentrations of the above genes were found between wastewater and river water samples collected in different seasons. 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). Its average concentration in river water sampled upstream and downstream from the wastewater discharge point was 1.6×10^2 gene copies/mL and 9.83×10^1 gene copies/mL, respectively. 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. This gene was also detected in treated wastewater in the concentration of 3.50×10^2 gene copies/mL, but only in the samples collected

in fall. The *van*A gene was detected in untreated wastewater samples in all seasons (8.28×10^5 gene copies/mL on average) and in treated wastewater in winter and summer (3.07×10^2 gene copies/mL on average). The *qac*A/B gene was identified in all samples excluding those from an activated sludge bioreactor, and its average concentration reached 3.83×10^5 gene copies/mL in untreated wastewater, 4.41×10^3 gene copies/mL in treated wastewater, 3.15×10^2 gene copies/mL in river water sampled upstream from the wastewater discharge point, and 4.37×10^3 gene copies/mL in river water sampled downstream from the wastewater discharge point.

Publication:

Zieliński W., Korzeniewska E., Harnisz M., Hubeny J., Buta M., Rolbiecki D. 2020. The prevalence of drug-resistant and virulent *Staphylococcus* spp. in a municipal wastewater treatment plant and their spread in the environment. Environment International 143, 105914. https://doi.org/10.1016/j.envint.2020.105914

Stage III

Aim: The aim of the third stage of the study was to perform a quantitative analysis of the concentrations of the genetic determinants of antibiotic resistance, the values of selected physicochemical parameters, the concentrations of selected antibiotics and HMs in samples of river water, wastewater and swabs collected from the upper respiratory tracts of WWTP employees. 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 was also determined.

Materials and Methods:

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. The examined WWTP relies on the activated sludge technology. It has a processing capacity of around 60 000 m^3, and receives mostly municipal and hospital (2%) wastewater. Samples were collected in three different seasons (winter, summer, fall) to account for potential differences in antibiotic consumption throughout the year. A total of 15 liquid samples (three samples from each sampling site) and six pooled swabs (three nasal swabs and three throat swabs) collected from 15 WWTP employees were analyzed. In addition, nasal and throat swabs were collected from the control group, i.e. healthy individuals who were not employed at the WWTP. Genomic DNA was isolated from the samples using the FastDNA

Spin Kit for Feces (MP Biomedicals), and qPCR was carried out to determine the presence and abundance of 23 ARGs, three genes encoding HM resistance and three integrase genes. The following physicochemical parameters were determined in the samples: total organic, inorganic and non-purgeable organic carbon, total nitrogen, chemical oxygen demand (COD), nitrogen forms and phosphorus forms, with the use of the Shimadzu TOC–L/TNM-L analyzer and Spectroquant® Test Kits (Merck, Poland). The samples were also analyzed to determine the concentrations with 16 antibiotics, using a HPLC system (Dionex Corporation, Sunyvale, CA, USA) coupled with an AB Sciex Q-Trap® 4000 mass spectrometer (Applied Biosystems/MDS SCIEX, Foster City, CA, USA). The concentrations of HMs (zinc, lead, cadmium, nickel, chromium, copper and arsenic) were determined by hydride generation-atomic absorption spectrometry (HGAAS) and inductively-coupled plasma optical emission spectroscopy (ICP-OES) in the last step of this stage of the study.

Results:

In the first part of the third stage of the study, qPCR was caried out to determine the concentrations of selected genes (17 ARGs, three genes encoding HM resistance and three integrase genes) in samples of URW and DRW, untreated wastewater, wastewater from an activated sludge bioreactor and treated wastewater, collected in three seasons. The obtained values varied significantly across the sampling seasons. Genes encoding resistance to various classes of popular antibiotics were examined in this experiment. All of the 17 analyzed ARGs were detected in at least one sample of genomic DNA from wastewater or river water. The highest concentrations of genes encoding resistance of beta-lactams, MLS antibiotics, sulfonamides and tetracycline were noted in wastewater samples. The concentrations of most ARGs decreased during successive stages of wastewater treatment, and only one out of the 17 analyzed ARGs (*tet*M) was not identified in treated wastewater. River water samples contained 13 out of the 17 analyzed ARGs, and the most prevalent genes were bla_{TEM} (1.02×10^8 gene copies /mL), *Amp*C (1.01×10^7 gene copies/mL), *sul*1 (2.62×10^5 gene copies/mL), *sul*2 (1.12×10^5 gene copies/ mL), *erm*F (4.36×10^5 gene copies/mL) and *tet*A (5.12×10^5 gene copies/mL). The concentrations of all ARGs were higher in DRW samples than in URW samples, and the noted differences were significant ($p < 0.05$). An analysis of the concentrations of class 1, 2 and 3 integron-integrase genes (*int*I1, *int*I2 and *int*I3) revealed that *int*I1 was most prevalent in samples of both river water and wastewater (2.62×10^4–1.17×10^6 gene copies/mL). The concentrations of the remaining two genes, *int*I2 and *int*I3, ranged from 3.81×10^4 to 5.62×10^4 gene copies/mL and from 1.53×10^3 to 5.55×10^4 gene copies/mL,

respectively. The last analysis in this part of the study was performed to determine the concentrations of genes encoding HM resistance. Only the *cnr*A gene, encoding microbial resistance to cobalt and nickel, was detected in river water and wastewater samples. The *cnr*A gene was not completely eliminated from wastewater because it was present in wastewater samples collected in each stage of the treatment process. This gene was also identified in URW (5.37×10^2–8.93×10^2 gene copies/mL) and DRW samples (1.2×10^3–1.36×10^3 gene copies/mL). The observed increase in the concentration of *cnr*A in DRW samples, compared with URW samples, was most probably caused by the discharge of effluents to the river.

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. The concentrations of most analyzed genes were higher in the DNA isolated from swabs collected from WWTP employees than in control group swabs, and the noted differences were significant ($p < 0.05$). *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), encoding resistance to MLS antibiotics, were most prevalent in throat swabs. *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 collected from WWTP employees. In the control group, the DNA isolated from upper respiratory tract specimens did not contain bla_{TEM}, *sul*1 or *int*I1; *erm*F, *mef*A and *Amp*C were detected in the DNA isolated from throat swabs, whereas *Amp*C was the only gene identified in the DNA isolated from nasal swabs. All 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.

In the next step of the third stage of the study, the values of selected physicochemical parameters were determined in wastewater and river water samples. Spearman's rank correlation coefficients were calculated based on the concentrations of chemical contaminants and the concentrations of the analyzed genes. The results were used to determine the potential effects of physicochemical parameters on the presence of antibiotic resistance determinants. The concentrations of total organic carbon, ammonia ions, total phosphorus and phosphate ions were positively correlated with the concentrations of numerous genes encoding resistance to different classes of antibiotics, and the strongest correlations were found for bla_{SHV} ($r = 0.81$–0.91), *mef*A ($r = 0.84$–0.91) and *aac(6')-Ib-cr* ($r = 0.86$–0.89). Positive correlations were also observed between the values of TOC, BOD, COD, NH_4, TP and PO_4 vs. the concentrations of the *cnr*A

gene encoding HM resistance (r = 0.86–0.91) and integrase genes (r = 0.78 –0.93). Strong positive correlations (r = 0.81–0.95) were also noted between the values of BOD and COD vs. the concentrations of the following ARGs: *bla*$_{OXA}$, *bla*$_{SHV}$, *sul*2, *mef*A, *aac(6')-Ib-cr* and *tet*A.

In the subsequent part of the study, the concentrations of selected antibiotics and HMs in river water and wastewater samples were analyzed to determine whether and to what extent they were eliminated in the examined WWTP with a mechanical biological treatment system. Antibiotic concentrations in river water and wastewater samples varied significantly depending on the sampling season ($p < 0.05$). Most of the identified antibiotics (except for sulfonamides and clindamycin) were present in higher concentrations in samples collected in fall, compared with those collected in winter and summer. Samples and untreated and treated wastewater contained 13 out 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, with a predominance of trimethoprim (12.12–1255.16 ng/L), ciprofloxacin (16.09–120.07 ng/L), clarithromycin (24.38–355.18 ng/L), sulfamethoxazole (22.26–69.41 ng/L) and doxycycline (7.0–78.70 ng/L). The concentrations of most analyzed antibiotics were higher in river water sampled downstream than upstream from the wastewater discharge point, and the observed differences were significant ($p < 0.05$). The concentrations of antibiotics in untreated and treated wastewater were analyzed to determine the efficacy of their removal during treatment. It was found that the effectiveness of drug removal during the technological process varied depending on antibiotic type and season. The concentrations of antibiotics were reduced to a greater extent in winter and summer than in fall. 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. A considerable reduction in clarithromycin concentration (82.6%) was observed only in samples collected in summer. In the next step of the third stage of the study, HM concentrations in river water and wastewater samples were analyzed, and no significant differences ($p < 0.05$) were noted across sampling seasons 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). Samples of wastewater from an activated sludge bioreactor contained all analyzed HMs, and zinc content (1.47–2.39 mg/L) exceeded the threshold limit (≤1 mg/L) in all samples.

In the subsequent part of the study, Spearman's rank correlation coefficients were calculated to find correlations between the experimental variables – the concentrations of integrase genes, ARGs, the gene encoding HM resistance, antibiotics and HMs, to evaluate the role of MGEs and co-selection mechanisms underlying HM-induced antibiotic resistance. Numerous significant ($p < 0.05$) positive correlations were noted between the concentrations of class 1, 2 and 3 integron-integrase genes and a wide array of ARGs. The strongest correlations were found between the concentrations of *mef*A and *int*I2 (r = 0.97), *erm*F and *int*I3 (r = 0.93), *sul*1 and *sul*2 vs. *int*I1 (r > 0.92), as well as between the concentrations of bla_{SHV}, bla_{OXA} and *aac(6')-Ib-cr* vs. *int*I2 (r = 0.90, r = 0.92 and r = 0.98, respectively). Strong positive correlations were also noted between the concentrations of *cnr*A and 12 ARGs (*erm*B, *erm*C, *erm*F, *mef*A, *sul*1, *sul*2, bla_{SHV}, bla_{OXA}, *tet*A, *aac(6')-Ib-cr*, *qep*A, *Amp*C) and three integrase genes (*int*I1, *int*I2, *int*I3). Apart from the co-existence of *cnr*A and ARGs, strong positive correlations were also observed between this gene and the concentrations of five out of the 16 analyzed antibiotics, i.e. ciprofloxacin (r = 0.75), clarithromycin (r = 0.70), sulfadiazine (r = 0.77), sulfamethoxazole (r = 0.8) and levofloxacin (r = 0.85). Significant positive correlations (r = 0.60–0.96) were also found between the concentrations of ARGs and HMs, in particular zinc and copper. Zinc concentration was bound by the strongest correlations with the abundance of *erm*B, *erm*C, *erm*F, *mef*A, *sul*1, *sul*2, bla_{SHV}, bla_{OXA}, *tet*A and *aac(6')-Ib-cr* genes, whereas copper concentration – with the abundance of *erm*C, *erm*F, *mef*A, *sul*1, *sul*2, bla_{SHV}, *tet*A, *aac(6')-Ib-cr* genes.

Publication:

Zieliński W., Korzeniewska E., Harnisz M., Drzymała J., Felis E., Bajkacz S. 2021. Wastewater treatment plants as a reservoir of integrase and antibiotic resistance genes – An epidemiological threat to workers and environment. Environment International 156, 106641. https://doi.org/10.1016/j.envint.2021.106641

Stage IV

Aim: In this stage of the study, the taxonomic composition of microorganisms was analyzed, and the resistome profile and mobility of genes were determined by metagenomic next-generation sequencing in samples of river water and wastewater, and upper respiratory tract specimens collected from WWTP employees. The main 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.

Materials and Methods:

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 were analyzed. The examined WWTP relies on a mechanical biological treatment system and the activated sludge technology, and it processes mainly municipal wastewater as well as small quantities of hospital wastewater. Samples were collected in three different seasons to account for potential differences in antibiotic consumption throughout the year. A total of six river water samples, nine wastewater samples and six pooled swabs collected from WWTP employees were analyzed. Liquid samples were passed through 0.2 μm filters; the filters and swabs were placed in test tubes containing phosphate-buffered saline (PBS) and were shaken; the resulting supernatant was used to isolate genomic DNA from wastewater samples and swabs – with the use of the FastDNA Spin Kit for Feces (MB Biomedicals), and from river water samples – with the use of the DNeasy PowerWater Kit (Qiagen). Whole-genome shotgun metagenomic sequencing was performed by Macrogen (Seoul, Korea), and DNA-seq libraries were prepared according to the TruSeq Nano DNA protocol. Metagenomic sequence data were subjected to detailed bioinformatics analysis. A total of 20 metagenomic samples were deposited in the NCBI database under accession No. PRJEB48483.

Results:

Metagenomic next-generation sequencing supported a detailed analysis of the taxonomic composition of microorganisms and their genetic profile in the examined samples of river water, wastewater and swabs collected from WWTP employees.

The first part of the fourth stage of the study focused on microbiome biodiversity in the samples, including differences between the sampling seasons. The structure of bacterial communities in wastewater samples collected during the three successive stages of the treatment process varied depending on the sampling season. Taxonomic diversity was highest in untreated wastewater samples collected in all seasons, and lowest in treated wastewater samples. The abundance of identified microbial phyla in samples of wastewater from an activated sludge bioreactor was identical in all seasons. In untreated wastewater samples, the predominant bacterial phyla were Proteobacteria (55.1%–93.6%), Firmicutes (2.7%–25.5%), Actinobacteria (1.9%–10.1%) and

Bacteroidetes (1.3%–6.4%). In samples of wastewater from an activated sludge bioreactor, the predominant bacterial phyla were Actinobacteria (38.6%–69%) and Proteobacteria (18.8%–36.4%), followed by lower percentages of Firmicutes (3.9%–16.6%), Bacteroidetes (5.6%), Nitrospirae (1.6%– 3.8%) and Acidobacteria (1.3%-2.8%). In treated wastewater samples, the predominant bacterial phyla were Proteobacteria (39%-82.7%), Actinobacteria (10.2%-26.2%) i Bacteroidetes (4.1%-19.6%), whereas the proportions of Firmicutes (0.4%-10%) and Verrucomicrobia (2.5%) were lower. In river water samples, the taxonomic composition of bacterial populations varied across sampling seasons. URW samples were characterized by lower biodiversity than DRW samples. In URW samples, Proteobacteria (95.4%) dominated in winter, Actinobacteria (52.1%) and Proteobacteria (43.8%) dominated in summer, whereas Proteobacteria (71.8%) and Cyanobacteria (25.6%) dominated in fall, with Actinobacteria accounting for only 1.8% of the bacterial community. In DRW samples collected in winter, the taxonomic composition of bacterial populations was similar to that observed in URW samples: Proteobacteria (92.8%) were the predominant phylum and the proportion of Actinobacteria reached 5.1%. In spring-summer samples, microbial composition was also similar to that noted in URW samples, with a predominance of Actinobacteria (48.8%) and Proteobacteria (44.2%). In fall, Cyanobacteria (58.5%) and Proteobacteria (31.1%) were most abundant, and the proportion of Actinobacteria reached 7.7%. The last taxonomic analysis involved upper respiratory tract specimens collected from WWTP employees. The structure of bacterial communities varied across sampling seasons. The phyla Firmicutes (51.9%) and Actinobacteria (47.7%) were predominant in nasal swabs collected in winter, whereas Actinobacteria (67.8%), Firmicutes (22.9%) and Proteobacteria (9%) were most abundant in nasal swabs collected in fall. Throat swabs collected in winter were characterized by a predominance of Firmicutes (33%), Actinobacteria (33.1%) and Bacteroidetes (28.9%). In summer, the predominant phyla were Actinobacteria (49.3%) and Firmicutes (37%), followed by Bacteroidetes (10.4%) and Proteobacteria (2.8%). Firmicutes (43%) dominated in throat samples collected in fall, followed by similar proportions of Bacteroidetes (24.9%) and Actinobacteria (21.5%). Bacterial communities identified in upper respiratory tract specimens are consistent with those reported in the literature, but a clear predominance of Actinobacteria in swabs and samples of wastewater from an activated sludge bioreactor may suggest that bioaerosol-borne Actinobacteria are transmitted to the upper respiratory tracts of WWTP employees during wastewater circulation and aeration in such bioreactors.

In the next step of the fourth stage of the study, the prevalence of ARGs was evaluated, including different types of antibiotics belonging to various classes. A total of 18 types of antibiotics were identified in all wastewater samples. Their relative abundance ranged from 2.88×10^{-5} gene copies/16S rRNA to 1.27 gene copies/16S rRNA 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; from 2.62×10^{-5} gene copies/16S rRNA copies to 7.3×10^{-1} gene copies/16S rRNA copies in TWW samples. In all stages of the wastewater treatment process, the predominant ARG types were multidrug resistance genes followed by genes encoding resistance to MLS antibiotics, beta-lactams, tetracyclines, aminoglycosides, sulfonamides and bacitracin. An analysis of changes in ARG concentrations in successive stages of wastewater treatment revealed that in each season, the concentrations of 11 ARG types were lowest in TWW samples, whereas the concentrations of six ARG types increased in TWW samples relative to UWW samples. River water samples contained 19 ARG types. Their relative abundance 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} gene copies/16S rRNA copies to 1.83 gene copies/16S rRNA copies in DRW samples. Samples of river water were characterized by the highest concentrations of multidrug resistance genes, followed by genes encoding resistance to beta-lactams, MLS antibiotics, aminoglycosides and bacitracin, whereas the diversity of the identified ARG types was higher in DRW samples than in URW samples. A total of 18 ARG types were identified in the swabs collected from WWTP employees. Their concentrations 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. The proportions of the predominant ARG types differed between nasal and throat swabs. Nasal swabs were characterized by the highest concentrations of genes encoding resistance to MLS drugs, aminoglycosides, chloramphenicol, sulfonamides, bacitracin as well as multidrug resistance genes, whereas genes encoding resistance to beta-lactams, tetracyclines and MLS antibiotics were most abundant in throat swabs.

The diversity of IS in river water samples, wastewater samples and swabs was analyzed in the subsequent step of the study. Insertion sequences are a large group of MGEs responsible for the transfer of ARGs between microorganisms. The proportions of the analyzed IS families were highly similar in wastewater samples collected in all stages of the treatment process in all seasons. The abundance of the examined IS families in wastewater samples ranged from 1,442.7 reads per kilobase (RPK) to 181,551.2 RPK in UWW samples; from 385.2 RPK to 13,700.9

RPK in AS samples; and from 455.6 RPK to 47,033.5 RPK in TWW samples. Families IS3, IS4, IS21 and IS66 were most abundant. The abundance of the examined IS families in river water samples ranged from 21.891 RPK to 43,486.325 RPK in URW samples, and from 99.5 RPK to 21,596 RPK in DRW samples. Similarly to wastewater samples, the most prevalent IS families in river water samples were IS3, IS4, IS21 and IS66 as well as IS5 and IS481. Different IS families were predominant in nasal and throat swabs collected from WWTP employees, but their RPK values were similar. The abundance of the identified IS families ranged from 36.554 RPK to 18,598.601 RPK in nasal swabs, and from 4.749 RPK to 17,133.497 RPK in throat swabs. In all seasons, families IS3, ISL3, IS30, IS6 and IS66 were predominant in nasal swabs, and families IS3, ISL3, IS30 and IS256 were most prevalent in throat swabs.

In the subsequent part of the study, the obtained data were analyzed in terms of the transmission of antibiotic resistance determinants and microorganisms with effluents to river water. The analysis included genes encoding resistance to the predominant types of antibiotics identified in the study. All ARGs selected for the comparative analysis were detected in TWW samples and in URW and DRW samples. The analysis revealed that the relative abundance of nearly all of the examined ARGs was higher in DRW than in URW samples. Moreover, DRW samples harbored ARGs that had not been detected in URW samples (including *sul*3, *qnr*S, $bla_{\text{OXA-1}}$ and *otr*A). In both URW and DRW samples, the most abundant ARGs were: *bac*A (log -1.22 to -0.94 gene copies/16S rRNA copies), *bcr*A (log -3.73 to -2.45 gene copies/16S rRNA copies), *emr*E (log -3.19 to -1.19 gene copies/16S rRNA copies), *mex*D (log -3.84 to -2.48 gene copies/16S rRNA copies), *aph(3")-I* (log -3.57 to -2.40 gene copies/16S rRNA copies), *sul*1 (log -3.71 to -2.43 gene copies/16S rRNA copies), *sul*2 (log -3.91 to -2.45 gene copies/16S rRNA copies), *tet*Q (log -3.98 to -2.87 gene copies/16S rRNA copies) and *erm*F (log -4.17 to -2.72 gene copies/16S rRNA copies). Insertion sequences were probably transmitted to river water because eight out of the 10 analyzed IS families (ISL3, IS4, IS6, IS21, IS30, IS66, IS256, IS481) were more abundant in DRW than in URW samples. An analysis of correlation coefficients revealed that the abundance of microorganisms which were most probably transmitted with effluents to river water was correlated with the concentrations of the previously analyzed ARGs and IS.

In the last step of the fourth stage of the study, the obtained data were analyzed in terms of the transmission of antibiotic resistance determinants to the upper respiratory tracts of WWTP employees. It was found that the relative abundance of some ARGs in nasal and throat samples was very similar to or higher than in wastewater samples. In nasal swabs, the dominant ARGs

were: *erm*C (log -1.75 to -0.80 gene copies/16S rRNA copies), *erm*X (log -1.21 to -0.73 gene copies/16S rRNA copies), *ant(2")-I* (log -3.43 to -1.55 gene copies/16S rRNA copies), *sul*1 (log -1.38 to -1.18 gene copies/16S rRNA copies) and *bac*A (log -1.49 to -1.25 gene copies/16S rRNA). In throat swabs, the most prevalent ARGs were: *cfx*A2 (log -1.72 to -1.33 gene copies/16S rRNA copies), *tet*M (log -2.47 to -1.71 gene copies/16S rRNA copies), *tet*Q (log -1.77 to -1.30 gene copies/16S rRNA copies), *erm*B (log -2.31 to -1.77 gene copies/16S rRNA copies) and *mef*A (log -1.58 to -1.21 gene copies/16S rRNA copies). An analysis of correlation coefficients revealed significant ($p < 0.05$) correlations between ARG concentrations and the abundance of the following bacterial phyla: Actinobacteria, Bacteroidetes, Proteobacteria, Firmicutes and Fusobacteria, which were identified in swabs collected from WWTP employees and in wastewater from an activated sludge bioreactor. Moreover, swabs collected from WWTP employees contained IS families (IS3, IS30, IS66 and ISL3) which, according to the literature, are not characteristic of the human microbiome, and which were detected in untreated wastewater samples and wastewater samples from an activated sludge bioreactor. An analysis of correlation coefficients also revealed that the abundance of the above IS families, in particular IS3 and IS30, was positively correlated with the presence of Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria phyla.

Publication:

Zieliński W., Hubeny J., Buta-Hubeny M., Rolbiecki D., Harnisz M., Paukszto Ł., Korzeniewska E. 2022. Metagenomics analysis of probable transmission of determinants of antibiotic resistance from wastewater to the environment – A case study. Science of the Total Environment 827, 154354. https://doi.org/10.1016/j.scitotenv.2022.154354

Summary and Conclusions

The study whose results constituted the basis for this doctoral dissertation expands the existing knowledge about the prevalence of ARB, ARGs, MGEs and chemical contaminants such as antibiotics and HMs in wastewater and river water. Conventional phenotypic methods for microbial identification as well as molecular biology techniques, physicochemical techniques and bioinformatics tools were used in the study. The research findings confirmed the hypothesis that WWTPs significantly contribute to the spread of ARB and antibiotic resistance determinants in the environment.

The results of this study indicate that WWTPs are a major source of ARB and pathogenic bacteria of the genus *Staphylococcus*. In consequence, effluents discharged to water bodies increase the abundance of staphylococci, antimicrobial resistance determinants and genes encoding pathogenicity in surface waters. A high degree of genetic similarity was observed between the strains obtained from wastewater and those isolated from WWTP employees, which may suggest that they can be potentially transmitted with the bioaerosol to the upper respiratory tracts of WWTP employees. The emission of these microorganisms to the air surrounding WWTPs poses a threat to public health, which should be closely monitored and evaluated in the future.

Moreover, the results of the study show that WWTPs are reservoirs of antibiotics, ARGs and MGEs. These micropollutants are not effectively eliminated in WWTPs that rely on the activated sludge technology. Thus, treated wastewater transmits antibiotics and their derivatives to the aquatic environment, which enriches the gene pool of environmental microorganisms in antibiotic resistance determinants. The present findings provide new insights into the transmission of antimicrobial resistance determinants and MGEs to surface water bodies, and their correlations. The current study demonstrated that the co-selection of antibiotic resistance may take place in the wastewater environment contaminated with HMs such as copper, zinc, chromium and arsenic. It was also found that ARGs can be transmitted with the bioaerosol emitted during wastewater treatments in activated sludge bioreactors and enter the respiratory system of WWTP employees with inhaled air, which may pose a threat to their health.

The study revealed that Actinobacteria predominated in wastewater sampled from an activated sludge bioreactor, which emits large quantities of bioaerosols, and in nasal and throat swabs collected from WWTP employees, and their abundance was strongly correlated with the concentrations of ARGs and IS. The observed relationships may point to their potential

transmission to the upper respiratory tracts of WWTP employees. This phenomenon can pose a potential health risk and can facilitate the spread of antibiotic resistance by HGT. The study also 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 research hypotheses were validated by the results of this study, conducted as part of a PhD program:

- Wastewater treated in WWTPs that rely on a mechanical biological treatment system and the activated sludge technology increases the abundance of antibiotic-resistant microorganisms and the genetic determinants of antibiotic resistance in water bodies receiving effluents.
- In WWTPs that rely on a mechanical biological treatment system and the activated sludge technology, antibiotic-resistant microorganisms and the genetic determinants of antibiotic resistance are transmitted, with the bioaerosol emitted during wastewater mixing and aeration, to the upper respiratory tracts of WWTP employees.

Bibliography

Beretsou, V.G., Michael-Kordatou, I., Michael, C., Santoro, D., El-Halwagy, M., Jäger, T., Besselink, H., Schwartz, T., Fatta-Kassinos, D., 2020. A chemical, microbiological and (eco)toxicological scheme to understand the efficiency of UV-C/H2O2 oxidation on antibiotic-related microcontaminants in treated urban wastewater. Sci. Total Environ. 744, 140835. https://doi.org/10.1016/j.scitotenv.2020.140835

Buta, M., Hubeny, J., Zieliński, W., Harnisz, M., Korzeniewska, E., 2021. Sewage sludge in agriculture – the effects of selected chemical pollutants and emerging genetic resistance determinants on the quality of soil and crops – a review. Ecotoxicol. Environ. Saf. 214. https://doi.org/10.1016/j.ecoenv.2021.112070

Byliński, H., Gębicki, J., Namieśnik, J., 2019. Evaluation of health hazard due to emission of volatile organic compounds from various processing units of wastewater treatment plant. Int. J. Environ. Res. Public Health 16. https://doi.org/10.3390/ijerph16101712

Cassini, A., Högberg, L.D., Plachouras, D., Quattrocchi, A., Hoxha, A., Simonsen, G.S., Colomb-Cotinat, M., Kretzschmar, M.E., Devleesschauwer, B., Cecchini, M., Ouakrim, D.A., Oliveira, T.C., Struelens, M.J., Suetens, C., Monnet, D.L., Strauss, R., Mertens, K., Struyf, T., Catry, B., Latour, K., Ivanov, I.N., Dobreva, E.G., Tambic Andraševic, A., Soprek, S., Budimir, A., Paphitou, N., Žemlicková, H., Schytte Olsen, S., Wolff Sönksen, U., Märtin, P., Ivanova, M., Lyytikäinen, O., Jalava, J., Coignard, B., Eckmanns, T., Abu Sin, M., Haller, S., Daikos, G.L., Gikas, A., Tsiodras, S., Kontopidou, F., Tóth, Á., Hajdu, Á., Guólaugsson, Ó., Kristinsson, K.G., Murchan, S., Burns, K., Pezzotti, P., Gagliotti, C., Dumpis, U., Liuimiene, A., Perrin, M., Borg, M.A., de Greeff, S.C., Monen, J.C., Koek, M.B., Elstrøm, P., Zabicka, D., Deptula, A., Hryniewicz, W., Caniça, M., Nogueira, P.J., Fernandes, P.A., Manageiro, V., Popescu, G.A., Serban, R.I., Schréterová, E., Litvová, S., Štefkovicová, M., Kolman, J., Klavs, I., Korošec, A., Aracil, B., Asensio, A., Pérez-Vázquez, M., Billström, H., Larsson, S., Reilly, J.S., Johnson, A., Hopkins, S., 2019. Attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: a population-level modelling analysis. Lancet Infect. Dis. 19, 56–66. https://doi.org/10.1016/S1473-3099(18)30605-4

Da Silva, M.F., Tiago, I., VerÃ-ssimo, A., Boaventura, R.A.R., Nunes, O.C., Manaia, C.M., 2006. Antibiotic resistance of enterococci and related bacteria in an urban wastewater treatment plant. FEMS Microbiol. Ecol. 55, 322–329. https://doi.org/10.1111/j.1574-6941.2005.00032.x

Dickinson, A.W., Power, A., Hansen, M.G., Brandt, K.K., Piliposian, G., Appleby, P., O'Neill, P.A., Jones, R.T., Sierocinski, P., Koskella, B., Vos, M., 2019. Heavy metal pollution and co-selection for antibiotic resistance: A microbial palaeontology approach. Environ. Int. 132, 105117. https://doi.org/10.1016/j.envint.2019.105117

Domínguez, M., Miranda, C.D., Fuentes, O., de la Fuente, M., Godoy, F.A., Bello-Toledo, H., González-Rocha, G., 2019. Occurrence of Transferable Integrons and sul and dfr Genes Among Sulfonamide-and/or Trimethoprim-Resistant Bacteria Isolated From Chilean Salmonid Farms. Front. Microbiol. 10. https://doi.org/10.3389/fmicb.2019.00748

ECDC, 2020. Antimicrobial consumption in the EU/EEA – Annual Epidemiological Report 2019. 1–25.

Felden, B., Cattoir, V., 2018. Bacterial Adaptation to Antibiotics through Regulatory RNAs.

Antimicrob. Agents Chemother. 62. https://doi.org/10.1128/AAC.02503-17

Felis, E., Buta-Hubeny, M., Zieliński, W., Hubeny, J., Harnisz, M., Bajkacz, S., Korzeniewska, E., 2022. Solar-light driven photodegradation of antimicrobials, their transformation by-products and antibiotic resistance determinants in treated wastewater. Sci. Total Environ. 836, 155447. https://doi.org/10.1016/j.scitotenv.2022.155447

Gaviria-Figueroa, A., Preisner, E.C., Hoque, S., Feigley, C.E., Norman, R.S., 2019. Emission and dispersal of antibiotic resistance genes through bioaerosols generated during the treatment of municipal sewage. Sci. Total Environ. 686, 402–412. https://doi.org/10.1016/j.scitotenv.2019.05.454

Ghaly, T.M., Geoghegan, J.L., Alroy, J., Gillings, M.R., 2019. High diversity and rapid spatial turnover of integron gene cassettes in soil. Environ. Microbiol. 21, 1567–1574. https://doi.org/10.1111/1462-2920.14551

Gogoi, H., Leiviskä, T., Heiderscheidt, E., Postila, H., Tanskanen, J., 2018. Removal of metals from industrial wastewater and urban runoff by mineral and bio-based sorbents. J. Environ. Manage. 209, 316–327. https://doi.org/10.1016/j.jenvman.2017.12.019

Grehs, B.W.N., Linton, M.A.O., Clasen, B., de Oliveira Silveira, A., Carissimi, E., 2021. Antibiotic resistance in wastewater treatment plants: understanding the problem and future perspectives. Arch. Microbiol. 203, 1009–1020. https://doi.org/10.1007/s00203-020-02093-6

Guo, M.T., Yuan, Q. Bin, Yang, J., 2013. Ultraviolet reduction of erythromycin and tetracycline resistant heterotrophic bacteria and their resistance genes in municipal wastewater. Chemosphere 93, 2864–2868. https://doi.org/10.1016/j.chemosphere.2013.08.068

Guo, X., Yan, Z., Zhang, Y., Xu, W., Kong, D., Shan, Z., Wang, N., 2018. Behavior of antibiotic resistance genes under extremely high-level antibiotic selection pressures in pharmaceutical wastewater treatment plants. Sci. Total Environ. 612, 119–128. https://doi.org/10.1016/j.scitotenv.2017.08.229

Han, Y., Li, L., Liu, J., 2013. Characterization of the airborne bacteria community at different distances from the rotating brushes in a wastewater treatment plant by 16S rRNA gene clone libraries. J. Environ. Sci. (China) 25, 5–15. https://doi.org/10.1016/S1001-0742(12)60018-7

Han, Y., Wang, Y., Li, L., Xu, G., Liu, J., Yang, K., 2018. Bacterial population and chemicals in bioaerosols from indoor environment: Sludge dewatering houses in nine municipal wastewater treatment plants. Sci. Total Environ. 618, 469–478. https://doi.org/10.1016/j.scitotenv.2017.11.071

Imran, M., Das, K.R., Naik, M.M., 2019. Co-selection of multi-antibiotic resistance in bacterial pathogens in metal and microplastic contaminated environments: An emerging health threat. Chemosphere 215, 846–857. https://doi.org/10.1016/j.chemosphere.2018.10.114

Jia, J., Guan, Y., Cheng, M., Chen, H., He, J., Wang, S., Wang, Z., 2018. Occurrence and distribution of antibiotics and antibiotic resistance genes in Ba River, China. Sci. Total Environ. 642, 1136–1144. https://doi.org/10.1016/j.scitotenv.2018.06.149

Karam, G., Chastre, J., Wilcox, M.H., Vincent, J.L., 2016. Antibiotic strategies in the era of

multidrug resistance. Crit. Care 20, 1–9. https://doi.org/10.1186/s13054-016-1320-7

Korzeniewska, E., Harnisz, M., 2013. Extended-spectrum beta-lactamase (ESBL)-positive Enterobacteriaceae in municipal sewage and their emission to the environment. J. Environ. Manage. 128, 904–911. https://doi.org/10.1016/j.jenvman.2013.06.051

Korzeniewska, E., Korzeniewska, A., Harnisz, M., 2013. Antibiotic resistant Escherichia coli in hospital and municipal sewage and their emission to the environment. Ecotoxicol. Environ. Saf. 91, 96–102. https://doi.org/10.1016/j.ecoenv.2013.01.014

Lekunberri, I., Balcázar, J.L., Borrego, C.M., 2018. Metagenomic exploration reveals a marked change in the river resistome and mobilome after treated wastewater discharges. Environ. Pollut. 234, 538–542. https://doi.org/10.1016/j.envpol.2017.12.001

Li, J., Zhou, L., Zhang, X., Xu, C., Dong, L., Yao, M., 2016. Bioaerosol emissions and detection of airborne antibiotic resistance genes from a wastewater treatment plant. Atmos. Environ. 124, 404–412. https://doi.org/10.1016/j.atmosenv.2015.06.030

Li, N., Sheng, G.P., Lu, Y.Z., Zeng, R.J., Yu, H.Q., 2017. Removal of antibiotic resistance genes from wastewater treatment plant effluent by coagulation. Water Res. 111, 204–212. https://doi.org/10.1016/j.watres.2017.01.010

Lopatkin, A.J., Huang, S., Smith, R.P., Srimani, J.K., Sysoeva, T.A., Bewick, S., Karig, D.K., You, L., 2016. Antibiotics as a selective driver for conjugation dynamics. Nat. Microbiol. 1, 1–22. https://doi.org/10.1038/nmicrobiol.2016.44

Maki, T., Hara, K., Iwata, A., Lee, K.C., Kawai, K., Kai, K., Kobayashi, F., Pointing, S.B., Archer, S., Hasegawa, H., Iwasaka, Y., 2017. Variations in airborne bacterial communities at high altitudes over the Noto Peninsula (Japan) in response to Asian dust events. Atmos. Chem. Phys. 17, 11877–11897. https://doi.org/10.5194/acp-17-11877-2017

Maraschin, M., Hedlund Ferrari, K.F., Carissimi, E., 2020. Acidification and flocculation of sludge from a water treatment plant: New action mechanisms. Sep. Purif. Technol. 252, 117417. https://doi.org/10.1016/j.seppur.2020.117417

Mazhar, S.H., Li, X., Rashid, A., Su, J.M., Xu, J., Brejnrod, A.D., Su, J.Q., Wu, Y., Zhu, Y.G., Zhou, S.G., Feng, R., Rensing, C., 2021. Co-selection of antibiotic resistance genes, and mobile genetic elements in the presence of heavy metals in poultry farm environments. Sci. Total Environ. 755. https://doi.org/10.1016/j.scitotenv.2020.142702

Minister of Maritime Economy and Inland Navigation. 2019. Regulation on substances particularly harmful to the aquatic environment and the conditions to be met when discharging sewage into waters or ground. as well as when discharging rainwater or meltwater into waters or into devices aquatic. Polish Journal of Laws, 1311.

Munita, J.M., Arias, C.A., 2016. Mechanisms of antibiotic resistance. Virulence Mech. Bact. Pathog. 4, 481–511. https://doi.org/10.1128/9781555819286.ch17

Osińska, A., Korzeniewska, E., Harnisz, M., Felis, E., Bajkacz, S., Jachimowicz, P., Niestępski, S., Konopka, I., 2020. Small-scale wastewater treatment plants as a source of the dissemination of antibiotic resistance genes in the aquatic environment. J. Hazard. Mater. 381, 121221. https://doi.org/10.1016/j.jhazmat.2019.121221

Partridge, S.R., Kwong, S.M., Firth, N., Jensen, S.O., 2018. Mobile Genetic Elements Associated with Antimicrobial Resistance. Clin. Microbiol. Rev. 31.

https://doi.org/10.1128/CMR.00088-17

Pazda, M., Kumirska, J., Stepnowski, P., Mulkiewicz, E., 2019. Antibiotic resistance genes identified in wastewater treatment plant systems – A review. Sci. Total Environ. 697, 134023. https://doi.org/10.1016/j.scitotenv.2019.134023

Proia, L., Von Schiller, D., Sànchez-Melsió, A., Sabater, S., Borrego, C.M., Rodríguez-Mozaz, S., Balcázar, J.L., 2016. Occurrence and persistence of antibiotic resistance genes in river biofilms after wastewater inputs in small rivers. Environ. Pollut. 210, 121–128. https://doi.org/10.1016/j.envpol.2015.11.035

Rensing, C., Moodley, A., Cavaco, L.M., McDevitt, S.F., 2018. Resistance to Metals Used in Agricultural Production. Microbiol. Spectr. 6. https://doi.org/10.1128/microbiolspec.ARBA-0025-2017

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M.C., Michael, I., Fatta-Kassinos, D., 2013. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. Sci. Total Environ. 447, 345–360. https://doi.org/10.1016/j.scitotenv.2013.01.032

Rizzo, L., Sannino, D., Vaiano, V., Sacco, O., Scarpa, A., Pietrogiacomi, D., 2014. Effect of solar simulated N-doped TiO2 photocatalysis on the inactivation and antibiotic resistance of an E. coli strain in biologically treated urban wastewater. Appl. Catal. B Environ. 144, 369–378. https://doi.org/10.1016/j.apcatb.2013.07.033

Roberto, A.A., Van Gray, J.B., Engohang-Ndong, J., Leff, L.G., 2019. Distribution and co-occurrence of antibiotic and metal resistance genes in biofilms of an anthropogenically impacted stream. Sci. Total Environ. 688, 437–449. https://doi.org/10.1016/j.scitotenv.2019.06.053

Silveira, E.O., Lutterbeck, C.A., Machado, Ê.L., Rodrigues, L.R., Rieger, A., Beckenkamp, F., Lobo, E.A., 2020. Biomonitoring of urban wastewaters treated by an integrated system combining microalgae and constructed wetlands. Sci. Total Environ. 705, 135864. https://doi.org/10.1016/j.scitotenv.2019.135864

Tytła, M., 2019. Assessment of heavy metal pollution and potential ecological risk in sewage sludge from municipal wastewater treatment plant located in the most industrialized region in poland—case study. Int. J. Environ. Res. Public Health 16, 1–16. https://doi.org/10.3390/ijerph16132430

Uhrbrand, K., Schultz, A.C., Madsen, A.M., 2011. Exposure to Airborne Noroviruses and Other Bioaerosol Components at a Wastewater Treatment Plant in Denmark. Food Environ. Virol. 3, 130–137. https://doi.org/10.1007/s12560-011-9068-3

WHO, 2014. Antimicrobial Resistance: Global Report on Surveillance. World Heal. Organ.

Yang, K., Li, L., Wang, Y., Xue, S., Han, Y., Liu, J., 2019. Airborne bacteria in a wastewater treatment plant: Emission characterization, source analysis and health risk assessment. Water Res. 149, 596–606. https://doi.org/10.1016/j.watres.2018.11.027