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DOCTORAL DISSERTATION

**Jakub Szymon Hubeny, MSc**

# Influence of wastewater origin on the spread of antibiotic resistance

Written under the guidance of

**Monika Harnisz**, Dr. Habil., Eng.,
Professor at the University of Warmia and Mazury in Olsztyn
**Sylwia Bajkacz**, Dr. Habil., Eng.,
Professor at the Silesian University of Technology

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ROZPRAWA DOKTORSKA

**mgr Jakub Szymon Hubeny**

# Wpływ pochodzenia ścieków na rozprzestrzenianie się antybiotykooporności

Pod kierunkiem

dr hab. inż. **Moniki Harnisz**,
profesor Uniwersytetu Warmińsko-Mazurskiego w Olsztynie
dr hab. inż. **Sylwii Bajkacz**,
profesor Politechniki Śląskiej

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5. 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. https://doi.org/10.1016/j.scitotenv.2022.155447. Number of points assigned by the Ministry of Education and Science: 200, IF: 10.753.

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*Appendix 1*

Hubeny, J., Buta, M., Zieliński, W., Harnisz, M., Korzeniewska, E., Nowrotek, M., Płaza, G., 2019. The prevalence of *tet*(A) and *tet*(M) tetracycline resistance genes in municipal wastewater. J. Ecol. Eng. 20, 1–6.

*Appendix 2*

Hubeny, J., Harnisz, M., Korzeniewska, E., Buta, M., Zieliński, W., Rolbiecki, D., Giebułtowicz, J., Nałęcz-Jawecki, G., Płaza, G., 2021. Industrialization as a source of heavy metals and antibiotics which can enhance the antibiotic resistance in wastewater, sewage sludge and river water. PLoS One 16, e0252691.

*Appendix 3*

Hubeny, J., Ciesielski, S., Harnisz, M., Korzeniewska, E., Dulski, T., Jałowiecki, Ł., Płaza, G., 2021. Impact of Hospital Wastewater on the Occurrence and Diversity of Beta-Lactamase Genes During Wastewater Treatment with an Emphasis on Carbapenemase Genes: A Metagenomic Approach. Front. Environ. Sci. 9.

*Appendix 4*

Hubeny, J., Korzeniewska, E., Buta-Hubeny, M., Zieliński, W., Rolbiecki, D., Harnisz, M., 2022. Characterization of carbapenem resistance in environmental samples and *Acinetobacter* spp. isolates from wastewater and river water in Poland. Sci. Total Environ. 822.

*Appendix 5*

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.

**Abstract**

Antibiotic resistance is a widespread problem that poses one of the greatest risks to public health around the world. The main cause of antibiotic resistance is the overuse of antibiotics in human and veterinary medicine, and in agriculture. Wastewater reaching wastewater treatment plants (WWTPs) contains antibiotics and/or heavy metals (HMs) that can induce selective pressure. Wastewater from urbanized and industrialized regions is characterized by higher concentrations of these pollutants than wastewater from less industrialized areas. In addition, hospital wastewater entering the sewerage system that conveys municipal wastewater to WWTPs also induces and promotes the spread of antimicrobial resistance determinants of particular clinical significance. Wastewater treatment plants are not equipped with appropriate technologies for eliminating genetic material from treated wastewater. A steady supply of genetic material, including antibiotic resistance genes (ARGs), to aquatic ecosystems turns them into an ideal environment for the acquisition and dissemination of ARGs due to the constant contamination of these ecosystems with antimicrobials of anthropogenic origin.

The aim of the study was to determine the effect of municipal wastewater of various origin on the antibiotic resistance of bacteria in wastewater and river water receiving effluents, and to evaluate the efficacy of photolysis affecting the degradation of antibiotics and antimicrobial resistance determinants in treated municipal wastewater.

The first stage of the study focused on the prevalence of tetracycline resistance genes in samples of wastewater, sludge and river water receiving effluents from two WWTPs differing in the characteristics of influents. The results revealed differences in the prevalence of ARGs depending on the type of influents. In the WWTP located in a tourism region with extensive forest cover, receiving 2% hospital wastewater and 8% industrial wastewater (WM-WWTP), the concentration of the *tet*(M) gene was not reduced during treatment. The *tet*(M) gene was detected on a seasonal basis in samples of activated sludge, treated wastewater, river water and sewage sludge. The research findings confirmed that ARGs cannot be effectively removed during the wastewater treatment process in WWTPs. In the other WWTP, located in an industrialized region and receiving 25% industrial wastewater and 0.4% hospital wastewater (S-WWTP), the analyzed genes were not eliminated during treatment. Seasonal changes in the prevalence of the analyzed genes were not observed. Tetracycline resistance genes were present in all samples of river water. The wastewater treatment process has to be modified to effectively reduce the concentrations of ARGs.

The aim of the second stage of the study was to evaluate the effect of different proportions of industrial and hospital wastewater on the development of antibiotic resistance in municipal wastewater treated in the examined WWTPs (S-WWTP receiving industrial wastewater and WM-WWTP receiving hospital wastewater). Correlations between pollutants such as antibiotics and HMs vs. antimicrobial resistance determinants, and the diversity of genes encoding resistance to beta-lactams, with particular emphasis on carbapenemase genes, during the treatment process were analyzed. Very few significant correlations were noted between the concentrations of antibiotics and ARGs in the examined samples. In turn, HM levels were correlated with ARG concentrations, which confirms the important role of HMs in promoting drug resistance. Significant ($p<0.05$) correlations were also found between the abundance of integrase genes, the abundance of ARGs and the concentrations of HMs. The samples from S-WWTP were characterized by higher HM contamination and a higher number of significant correlations between the analyzed variables than the samples from WM-WWTP. A comparison of wastewater samples from two WWTPs receiving different amounts of hospital wastewater revealed higher diversity of genes encoding resistance to beta-lactams in the samples with a higher proportion of hospital wastewater (58 types) than in those with a lower proportion of hospital wastewater (52 types). In samples from both WWTPs, the dominant types of beta-lactamase genes were *bla*$_{\text{OXA}}$, *bla*$_{\text{GES}}$, *bla*$_{\text{BEL}}$, *bla*$_{\text{CfxA}}$ and *bla*$_{\text{TEM}}$. It is worth noting that *bla*$_{\text{KPC}}$ and *bla*$_{\text{NDM}}$ were detected only in untreated municipal wastewater with a higher proportion of hospital wastewater. The number of correlations between the abundance of beta-lactamases and integrase genes was 94 in wastewater with a higher proportion of hospital wastewater and 41 in wastewater with a lower proportion of hospital wastewater. Therefore, the discharge of effluents from WWTPs to the natural environment may pose a constant threat to human life and health due to the transfer of pollutants such as ARGs, antibiotics and HMs.

The third stage of the study focused on determining the presence of antimicrobial resistance determinants in *Acinetobacter* sp. strains isolated from wastewater samples in each stage of the treatment process, and in river water samples. Subsequently, molecular techniques such as qualitative and quantitative PCR and metagenomic analysis were used to detect the presence of beta-lactam resistance genes (Ambler class B and D), insertion sequence *ISAba1*, class 1, 2 and 3 integron-integrase genes and the taxonomic structure of bacteria. The detection frequency of *Acinetobacte*r sp. and *A. baumannii* was highest in wastewater and river water samples collected in June and September. In the group of resistance genes, *bla*$_{\text{OXA-51}}$ was most prevalent in *Acinetobacter* strains. However, metagenomic analysis demonstrated that *bla*$_{\text{OXA-58}}$ was most abundant in the studied samples. The IS*Aba1*/*bla*$_{\text{OXA-51}}$ complex was identified in

13 strains, which confirmed the occurrence of carbapenem-resistance strains in the analyzed samples. The fact that the number of *Acinetobacter* isolates carrying ARGs was not reduced in treated wastewater compared with untreated wastewater suggests that the treatment process was ineffective.

The aim of the fourth stage of the study was to determine the efficacy of removing antibiotics and antimicrobial resistance determinants from treated wastewater by photodegradation. It was found that solar light combined with a photocatalyst ($TiO_2$) significantly decreased the concentrations of antibiotics and ARGs in wastewater. The tested methods supported the elimination of 52% to 69% of antibacterial agents, and 84% to 90% of the analyzed genes. The incorporation of the above methods to the wastewater treatment system may significantly improve the quality of effluents discharged to the natural environment.

The research findings indicate that the composition of wastewater treated in WWTPs, determined by its origin, significantly affects the development of antibiotic resistance. The number of significant correlations between HMs and antimicrobial resistance determinants was higher in the samples from a WWTP located in a highly industrialized region characterized by high HM contamination than in the samples from a WWTP located in a less industrialized area. It was found that HMs induce antibiotic resistance. In turn, the prevalence of ARGs of particular clinical significance was higher in wastewater samples from a WWTP receiving a higher proportion of hospital wastewater (2%) than in those from a WWTP receiving a lower proportion of hospital wastewater (0.4%). Finally, it was demonstrated that solar-light driven photodegradation used for removing micropollutants from treated wastewater significantly contributes to preventing the spread of antibiotic resistance due to effective antibiotic degradation and a decrease in the abundance of antimicrobial resistance determinants in wastewater.

**Streszczenie**

Obecnie ludzie na całym świecie są zmuszeni stawić czoła zjawisku antybiotykooporności. Ten szeroko rozpowszechniony problem stanowi dzisiaj jedno z największych zagrożeń zdrowia publicznego, wynikających głównie z nadużywania antybiotyków w medycynie, weterynarii oraz rolnictwie. Ścieki dopływające do oczyszczalni ścieków (WWTPs) zawierają antybiotyki i/lub metale ciężkie (HMs), które mogą być czynnikami wywołującymi presję selekcyjną. Ścieki z regionów o wyższym stopniu urbanizacji oraz uprzemysłowienia charakteryzują się wyższą koncentracją tych zanieczyszczeń w porównaniu do ścieków z regionów słabiej uprzemysłowionych. Dodatkowo, dopływ ścieków szpitalnych do sieci kanalizacyjnej doprowadzającej ścieki komunalne do WWTP również stanowi bardzo istotny czynnik indukujący rozprzestrzenianie determinantów antybiotykooporności o znaczeniu klinicznym. Oczyszczalnie ścieków nie posiadają systemów, które eliminowałyby materiał genetyczny ze ścieków. Poprzez stały napływ materiału genetycznego, w tym genów antybiotykooporności (ARGs), ekosystemy wodne stanowią idealne środowisko do nabywania i rozprzestrzeniania ARGs z powodu ciągłego zanieczyszczenia tych ekosystemów związkami przeciwdrobnoustrojowymi pochodzenia antropogenicznego.

Celem pracy było określenie wpływu pochodzenia ścieków komunalnych na antybiotykooporność bakterii bytujących w ściekach i wodzie rzecznej będącej odbieralnikiem ścieków oczyszczonych oraz określenie efektywności procesu fotolizy wpływającej na degradacje antybiotyków i determinantów antybiotykooporności w oczyszczonych ściekach komunalnych.

W pierwszym etapie badań skupiono się na występowaniu genów oporności na tetracykliny w próbkach ścieków, osadów ściekowych oraz wody rzecznej będącej odbiornikiem ścieków oczyszczonych z dwóch WWTPs różniących się charakterystyką ścieków dopływających. Wyniki wskazują na występujące różnice w częstości pojawiania się genów oporności w zależności od charakterystyki ścieków dopływających. WWTP zlokalizowana w regionie wysoce turystycznym z bogatym zalesieniem i charakteryzującej się 2%-wym dopływem ścieków szpitalnych oraz 8% dopływem ścieków przemysłowych (WM-WWTP), wykazano brak redukcji występowania genu *tet*(A) po procesie oczyszczania. W przypadku genu *tet*(M) odnotowano sezonowe zmiany jego obecności, a mianowicie w próbkach osadu czynnego, ścieków oczyszczonych, wody rzecznej oraz osadów ściekowych. Wykonane badania potwierdzają brak możliwości usuwania ARGs w procesie technologicznym oczyszczania ścieków wykorzystanym przez WWTPs. W drugiej WWTP

zlokalizowanej w regionie zindustrializowanym z 25%-wym udziałem dopływających ścieków przemysłowych oraz 0.4%-wym udziałem dopływających ścieków szpitalnych (S-WWTP), cechowała się brakiem eliminacji genów badanych po procesie oczyszczania. Nie zaobserwowano tam również zmian sezonowych występowania badanych genów. Ponadto geny z grupy tetracyklin były obecne we wszystkich próbkach wody rzecznej. Do wydajnego redukowania genów antybiotykooporności ze ścieków wymagana jest modyfikacja procesu oczyszczania.

Celem drugiego etapu badań była ocena wpływu udziału ścieków industrialnych oraz szpitalnych na zjawisko antybiotykooporności w ściekach komunalnych oczyszczanych w badanych WWTPs (S-WWTP powiązanej z dopływem ścieków przemysłowych oraz WM-WWTP powiązanej z dopływem ścieków szpitalnych). Do tego celu wykorzystano wyniki z przeprowadzonych analiz zależności pomiędzy zanieczyszczeniami takimi jak antybiotyki i HMs a determinantami antybiotykooporności oraz wyniki określające różnorodność genów warunkujących oporność na antybiotyki beta-laktamowe, ze szczególnym uwzględnieniem genów karbapenemaz, podczas procesu oczyszczania. Zaobserwowano niewiele istotnych zależności pomiędzy koncentracją antybiotyków w badanych próbkach a występowaniem w nich ARGs. Z kolei stężenie HMs korelowało z koncentracją ARGs, co potwierdza istotną rolę HMs w promowaniu występowania zjawiska lekooporności. Zauważono również występowanie istotnych zależności ($p<0.05$) pomiędzy liczebnością genów integrazy a liczebnością ARGs oraz koncentracją HMs. Próbki z S-WWTP charakteryzowały się wyższym zanieczyszczeniem metalami ciężkimi oraz większą ilością istotnie statystycznych korelacji pomiędzy badanymi zmiennymi w porównaniu do próbek pochodzących z WM-WWTP. Porównując próbki ścieków z dwóch oczyszczalni charakteryzujących się dopływem ścieków szpitalnych na różnym poziomie, stwierdzono większą różnorodność typów genów warunkujących oporność na beta-laktamy w ściekach z wyższym udziałem procentowym ścieków szpitalnych (58 typów) w porównaniu do ścieków z mniejszym udziałem (52 typy). W próbkach z obu oczyszczalni dominującymi genami beta-laktamaz były typy $bla_{\mathrm{OXA}}$, $bla_{\mathrm{GES}}$, $bla_{\mathrm{BEL}}$, $bla_{\mathrm{CfxA}}$ oraz $bla_{\mathrm{TEM}}$. Warto zwrócić uwagę, że geny $bla_{\mathrm{KPC}}$ oraz $bla_{\mathrm{NDM}}$ zostały stwierdzone tylko w nieoczyszczonych ściekach komunalnych o większej zawartości ścieków szpitalnych. W ściekach charakteryzujących się wyższym udziałem ścieków szpitalnych zaobserwowano 94 korelacje, podczas gdy w ściekach z mniejszym udziałem zanotowano 41 korelacji, pomiędzy częstotliwością występowania genów integrazy oraz genów beta-laktamaz. Zrzut ścieków oczyszczonych z WWTPs do środowiska naturalnego może stanowić zatem

ciągłe zagrożenie życia i zdrowia ludzi, poprzez przenoszenie zanieczyszczeń takich jak geny antybiotykooporności, antybiotyki czy HMs do środowiska.

W badaniach prowadzonych w trzecim etapie skupiono uwagę na określeniu obecności determinantów oporności na antybiotyki u szczepów *Acinetobacter* sp. wyizolowanych z próbek ścieków w poszczególnych etapach procesu oczyszczania oraz próbek wody rzecznej. Następnie, przy użyciu narzędzi molekularnych, takich jak jakościowa i ilościowa reakcja PCR oraz analiza metagenomiczna, określono obecność genów oporności na antybiotyki beta-laktamowe (klasy B i D Amblera), sekwencji insercyjnej *ISAba*1, genów integrazy klasy I, II i III oraz strukturę taksonomiczną. Szczepy *Acinetobacte*r sp. oraz *A. baumannii* najczęściej określano w próbkach ścieków i wody rzecznej pobranych w czerwcu i wrześniu. Wśród analizowanych genów oporności, najwyższą częstością występowania u szczepów *Acinetobacter* sp. wykazywał gen $bla_{\text{OXA-51}}$, natomiast analiza metagenomiczna wskazała, że gen $bla_{\text{OXA-58}}$ występował najliczniej w badanych próbkach. W obrębie 13 szczepów stwierdzono obecność kompleksu *ISAba1*/$bla_{\text{OXA-51}}$, co potwierdza występowanie szczepów opornych na karbapenemy w analizowanych próbkach. Brak redukcji liczby izolatów *Acinetobacter* sp. niosących geny oporności na antybiotyki w próbkach ścieków oczyszczonych w porównaniu do ścieków dopływających sugeruje, że procesy oczyszczania stosowane w oczyszczalni ścieków są nieefektywne.

Celem czwartego etapu było określenie efektywności usuwania antybiotyków oraz determinantów antybiotykooporności ze ścieków oczyszczonych wskutek zastosowania procesu fotodegradacji. Wykazano, że zastosowane naświetlania światłem słonecznym oraz wykorzystanie dodatkowo fotokatalizatora ($TiO_2$) istotnie wpływa na zmniejszenie ilości antybiotyków oraz ARGs w ściekach. Wykazano, że analizowane metody są w stanie wyeliminować od 52% do 69% badanych związków antybakteryjnych oraz od 84% do 90% badanych genów. Wdrożenie tych metod do ciągu technologicznego oczyszczania ścieków może powodować istotne poprawienie jakości ścieków oczyszczonych wprowadzanych do środowiska naturalnego.

Biorąc pod uwagę zebrane wyniki, można stwierdzić, że skład ścieków oczyszczanych w WWTPs, kreowany poprzez ich pochodzenie, istotnie wpływa na zjawisko antybiotykooporności. W próbkach pochodzących z WWTP zlokalizowanej w regionie wysoce uprzemysłowionym charakteryzującym się wysokim zanieczyszczeniem HMs, określono więcej istotnych zależności pomiędzy HMs a determinantami oporności niż w próbkach uzyskanych z WWTP zlokalizowanej w regionie o mniejszym uprzemysłowieniu. Z przeprowadzonych badań wynika, że HMs indukującą problem antybiotykooporności.

Natomiast w ściekach pobranych z WWTP mającej większy dopływ ścieków szpitalnych (2%), w porównaniu do WWTP mającej mniejszy udział owych ścieków (0.4%), zaobserwowano częstsze występowanie genów oporności o znaczeniu istotnym klinicznie. Ostatecznie, stwierdzono, że wykorzystanie procesów fotokatalizy oraz fotodegradacji w usuwaniu mikrozanieczyszczeń ze ścieków oczyszczonych istotnie wpływa na ograniczenie rozprzestrzeniania zjawiska oporności poprzez degradację antybiotyków oraz redukcję liczebności występujących w ściekach determinantów oporności.

## Introduction

Antibiotic resistance is widely reported around the world, and the development of and rapid changes in the bacterial resistome pose a significant problem. The emergence of drug resistance can be attributed to overpopulation, intensified migration, antibiotic overuse in human and veterinary medicine and in livestock farming, and ineffective wastewater treatment systems. Antibiotics are the mainstays of modern medicine, and they are administered to treat bacterial infections (Singer et al., 2016). The first historical records describing treatments for bacterial infections date back to ancient Egypt and Greece. The modern antibiotic era began in 1928 with the discovery of penicillin by Alexander Fleming. Since then, antibiotics have saved millions of lives. Antibiotics have been prescribed to treat bacterial infections already in the 1940s, but the widespread use of antibiotics and growing levels of antimicrobial resistance emerged a significant clinical problem already in the following decade (Ventola, 2015). The golden era of antibiotics lasted from the 1930s to the 1960s, and numerous antimicrobials were developed during that period (Nathan and Cars, 2014). However, this era came to an end because scientists were unable to keep up with the rapid emergence of drug-resistant pathogens. The failure to design or discover new antibiotics, and antibiotic misuse and overuse contribute to antimicrobial resistance (Wang et al., 2018). The evolution of resistance mechanisms is fueled by microbial competition for resources, including the production of secondary metabolites which resemble many antibiotics (Larsson and Flach, 2022).

Horizontal gene transfer (HTG) is the key mechanism responsible for the acquisition of antibiotic resistance by bacteria. This process enables bacterial cells to exchange genetic material. Antibiotic resistance genes (ARGs) can be distributed by plasmids, transposons, and integrons which act as vectors that transmit genetic information. Antimicrobial resistance can be also acquired through chromosome mutations. However, these mechanisms differ in the rate of resistance acquisition, to the advantage of HGT (Hiltunen et al., 2017). Wide use of antibiotics induced radical changes in evolutionary forces, promoted the spread of resistance, and exerted unprecedented selection pressure, in particular on human microbiota, but also on microorganisms colonizing environments that are heavily polluted with antibiotics or HMs. In numerous bacterial species, selection pressure induced the activation and exchange of various ARGs via HGT (Alcock et al., 2019). These evolutionary events have contributed to a worldwide antibiotic resistance crisis and a shortage of effective antimicrobials for treating bacterial infections. According to the One Health concept, the links between human, animal and environmental microbiota need to be explored to resolve the global antibiotic crisis because

antibiotic-resistant bacteria (ARB) and ARGs can spread across sectors, settings, and geographic boundaries (Andersson et al., 2020; Mackenzie and Jeggo, 2019).

Antimicrobials actively target pathogens and are invaluable in the treatment of bacterial infections. However, human activities have enabled microorganisms to acquire resistance mechanisms that neutralize the effects of antibiotics (Berglund, 2015). According to a 2020 report of the European Center of Disease Prevention and Control (ECDC), the average consumption of antibiotics in Europe was determined at 19.4 defined daily dose (DDD) per 1000 inhabitants. In the European Union (EU), antibiotic consumption is highest in Greece and lowest in the Netherlands, whereas Poland ranks 11$^{th}$ in this respect (23.6 DDD) and exceeds the EU/EEA average (Buta et al., 2021). Antibiotics are partly or completely metabolized by human and veterinary patients. They are excreted with feces and urine in active or inactive form, and they often reach WWTPs. Antibiotic concentrations are reduced during wastewater treatment. Drugs that are not removed in the wastewater treatment process reach the environment with effluents (Reijnders et al., 2016).

The continuous presence of antibiotics in a given environment leads to the proliferation and spread of ARB and ARGs in various ecosystems (Lopatkin et al., 2016). Both ARB and ARGs are ubiquitous in the natural environment, but human activities significantly contribute to their emergence and transmission. Wastewater treatment plants are regarded as significant hotspots of antibiotic resistance (Berendonk et al., 2015) and direct contributors to the spread of antimicrobial resistance in the natural environment (Di Cesare et al., 2016b). The spread of ARGs in the environment directly affects public health. According to the literature, environmental bacteria promote the emergence of antibiotic resistance in clinical strains (Xiong et al., 2015). Human pathogens can also acquire ARGs in the natural environment (D'Costa et al., 2011). Organisms that belong to different kingdoms and colonize the same habitat harbor numerous microorganisms that share the same pool of ARGs. Wastewater treatment plants process effluents containing antibiotics, HMs, ARB, and ARGs from hospitals, households, and industrial plants. Wastewater treatment systems are designed to remove organic compounds and suspended solids, and they effectively reduce bacterial cell counts, but none of the existing solutions are capable of eliminating genetic material, including mobile genetic elements (MGEs) (Wang et al., 2017).

The composition of municipal wastewater is determined by various streams of influents that reach WWTPs from households, industrial plants, hospitals, and rainwater collectors. Wastewater pollution levels can differ across regions. In highly industrialized regions, effluents are more likely to be contaminated with HMs (Di Cesare et al., 2016a). Heavy metals are among

the most toxic components in wastewater. These pollutants are not degraded, and they are strongly immobilized by solid particles such as sewage sludge (Tytła, 2019). The chemical composition of industrial wastewater can affect the ARG profile of microbial communities and, in particular, it can be responsible for high HM concentrations. In bacteria, metal resistance is directly correlated with antibiotic resistance. Bacteria can co-select for metal-driven antibiotic resistance under stress (Fiorentino et al., 2019). Three types of co-selection mechanisms have been identified: co-occurrence, co-resistance, and co-regulation (Mazhar et al., 2021). The proportion of hospital wastewater in municipal wastewater is yet another important factor affecting the resistance profile in influents reaching WWTPs. Hospital wastewater is one of the main sources of ARB, ARGs, and antibiotics (Seruga Music et al., 2017). High concentrations of antibiotic residues in wastewater can exert selective pressure on ARGs and promote the spread of antibiotic resistance (Zheng et al., 2018). Hospital wastewater is a major source of pathogens that are resistant to the most common classes of antibiotics. These pathogens include carbapenemase-producing bacteria, extended-spectrum beta-lactamase-producing *Enterobacteriaceae*, and bacteria harboring vancomycin resistance determinants (Hassoun-Kheir et al., 2020). In addition, due to the much higher use of antibiotics in hospitals, the ARG profile of hospital wastewater can differ from that noted in other types of wastewater. This difference further increases the risk of spreading resistance to antibiotics used in the hospital setting, including last-resort antibiotics (Zhang et al., 2020). Hospital wastewater is characterized by high ARB loads around the world, but only several countries recommend that hospital wastewater is treated before it is discharged to receiving water bodies. According to Council Directive (91/271/EEC), hospital wastewater does not have to be treated before it is evacuated to the municipal sewage system and WWTPs (Hocquet et al., 2016).

In conclusion, wastewater from various sources can differ in composition, and it can directly or indirectly contribute to the emergence and spread of antibiotic resistance. Therefore, wastewater should be regularly monitored to control the concentrations of antimicrobial resistance determinants reaching the natural environment.

## Research objective, research hypotheses and scope of the study

### Research objective

The aim of the study was to determine the effect of municipal wastewater of various origin on the antibiotic resistance of bacteria in wastewater and river water receiving effluents, and to evaluate the effectiveness of photolytic and photocatalytic methods in removing antibiotics and antimicrobial resistance determinants from treated municipal wastewater.

### Research hypotheses

1. Industrialization and the proportion of hospital wastewater in municipal wastewater affect the structure of resistome in municipal wastewater treated in a WWTP.
2. WWTPs that rely on a mechanical biological system based on the activated sludge technology significantly contribute to the spread of micropollutants in the natural environment.
3. The processes of photolysis and photocatalysis significantly contribute to the removal of pollutants promoting antibiotic resistance.

### Scope of the study

1. A preliminary analysis of the effect of wastewater from two regions with different levels of industrialization on the diversity of antimicrobial resistance determinants in the tested samples (research article in Appendix No. 1 to this dissertation).
2. Determination of the impact of industrialization in the region where municipal wastewater samples were collected, and the proportion of hospital wastewater in municipal wastewater flowing into WWTPs on antibiotic concentrations and the structure of antimicrobial resistance determinants in treated wastewater and river water receiving effluents (research articles in Appendices No. 2 and 3 to this dissertation).
3. Determination of the effect of hospital wastewater on the antibiotic resistance profile and environmental contamination with potentially pathogenic bacteria – a detailed analysis of public health threats (research article in Appendix No. 4 to this dissertation).
4. Determination of the removal efficiency of antibiotics and antimicrobial resistance determinants from treated wastewater with the use of photolysis and photocatalysis (research article in Appendix No. 5 to this dissertation)

## Results

The presented doctoral research was conducted in four stages, and the results were described in five research articles. The first three stages focused on the effect of different proportions of industrial and hospital wastewater in municipal wastewater on the antibiotic resistance profile and the taxonomic structure of microorganisms in wastewater and river water receiving effluents. The fourth stage focused on the removal efficiency of antibiotics and antimicrobial resistance determinants from treated wastewater with the use of photolysis and photocatalysis in the presence of $TiO_2$.

### Stage I

*Aim of the study:*

The aim of the first stage of the study was to determine the prevalence of tetracycline resistance genes in samples of wastewater, sludge and river water receiving effluents.

*Materials and Methods:*

Samples were collected twice, in June and November 2018, in two Polish regions with different levels of industrialization. The first region is characterized by extensive forest cover, tourism and recreation. The second region is characterized by high levels of urbanization and industrialization. One WWTP from each region was selected for the study. Wastewater and sludge samples were collected in selected stages of the treatment process in both WWTPs. Samples of untreated wastewater, wastewater after primary sedimentation, wastewater treated in a biological reactor, wastewater treated in a multifunctional reactor, treated wastewater, sludge from the open fermentation tank and dewatered sludge prepared for disposal were collected in the WWTP located in the first region (WM-WWTP). Samples of untreated wastewater, wastewater after primary and secondary sedimentation, wastewater treated in a C-TECH reactor, treated wastewater, dewatered sludge, mechanically compacted sludge and sludge after gravity thickening were collected in the WWTP located in the second region. Samples of river water were collected upstream and downstream from the effluent discharge point. Samples of wastewater and river water were filtered with a vacuum pump (MilliPore, Merck) with polycarbonate membrane filters with 0.2 µm porosity. Sludge samples were not filtered and were used directly for DNA isolation. Genomic DNA was isolated from wastewater and river water with the DNeasy Power Water Kit (Qiagen), and from sewage sludge with the

DNeasy Power Soil Kit (Qiagen). The presence of tetracycline resistance genes (*tet*(M) and *tet*(A)) in the genomic DNA of samples was determined in a PCR assay.

*Results:*

The study revealed the absence of differences in the prevalence of the *tet*(A) gene. It was identified in all samples from all sites in both WWTPs and sampling seasons. The *tet*(M) gene was identified in all samples from S-WWTP. The *tet*(M) gene was not detected in samples of treated wastewater or sludge collected in fall in WM-WWTP. The *tet*(M) gene was not found in samples of river water in summer or fall, either. The absence of the *tet*(M) gene in samples of river water collected upstream from WM-WWTP could point to low concentrations of this gene in the environment. In turn, the absence of the *tet*(M) gene in samples of river water collected downstream from WM-WWTP could result from too low concentrations of the *tet*(M) gene that were below the detection threshold due to the dilution of discharged effluents and self-purification of river water.

*Publication:*

**Hubeny, J.**, Buta, M., Zieliński, W., Harnisz, M., Korzeniewska, E., Nowrotek, M., Płaza, G., 2019. The prevalence of *tet*(A) and *tet*(M) tetracycline resistance genes in municipal wastewater. J. Ecol. Eng. 20, 1–6. https://doi.org/10.12911/22998993/112714.

Number of points assigned by the Ministry of Education and Science: 40

Stage II

*Aim of the study:*

The aim of the second stage of the study was to evaluate the effect of different proportions of industrial and hospital wastewater on the development of antibiotic resistance in municipal wastewater treated in the examined WWTPs and in river water receiving effluents. The evaluation was based on: 1) analyses of the concentrations of and correlations between pollutants present in the tested samples, such as antibiotics, HMs and antimicrobial resistance determinants, and 2) analyses of the abundance and diversity of genes encoding resistance to beta-lactams, with particular emphasis on carbapenemase genes. Samples were collected in two WWTPs receiving hospital and industrial wastewater in different proportions.

*Materials and Methods:*

Samples of wastewater, sludge and river water, collected in June and November as described in the first stage of the study, were analyzed. Samples collected in the same sampling sites in March (2019) were also analyzed. Samples were obtained from two WWTPs receiving hospital and industrial wastewater in different proportions. The first WWTP (WM-WWTP) received 2% hospital wastewater and 8% industrial wastewater, and the second WWTP (S-WWTP) received 0.4% hospital wastewater and 25% industrial wastewater. Environmental DNA was extracted from wastewater and river water using the Power Water (MoBio Laboratories Inc., CA, USA) kit, and from sewage sludge using the Power Soil kit (MoBio Laboratories Inc., CA, USA).

The results obtained in this stage of the study were published in two research articles. In the study described in the first article (research article in Appendix No. 2 to this dissertation), quantitative PCR was used to analyze the concentrations of selected genes encoding resistance to beta-lactams (*bla*$_{TEM}$, *bla*$_{OXA}$, *bla*$_{SHV}$), tetracyclines (*tet*(A), *tet*(M)), fluoroquinolones (*qep*A, *aac*(6`)-*Ib-cr*), sulfonamides (*sul*1, *sul*2), integrase genes (*int*I1, *int*I2), and the 16S rRNA gene. The concentrations of six HMs (Zn, Pb, Ni, Cr, Co and As) and nine antibiotics of the following groups: 1) tetracyclines (oxytetracycline (OTC) and tetracycline (TET)), 2) sulfonamides (sulfadimethoxine (SDM), sulfamethoxazole (SXT), and sulfathiazole (ST)), 3) fluoroquinolones (ciprofloxacin (CIP), norfloxacin (NOR), pefloxacin (PEF), and ofloxacin (OFX)) were also determined. In the study described in the second article (research article in Appendix No. 3 to this dissertation), metagenomic analysis was performed to determine the presence and concentrations of genes encoding resistance to beta-lactams, with particular emphasis on carbapenem resistance genes (CRGs), and taxonomic structure.

*Results:*

In the study whose results were published in the research article in Appendix No. 2 to this dissertation, quantitative PCR supported the identification of nine ARGs, two integrase genes and the 16S rRNA gene in the analyzed samples. The concentrations of ARGs and integrase genes ranged from $10^1$ - $10^9$ gene copies/mL in wastewater to $10^3$ - $10^{11}$ gene copies/g in sewage sludge.

Genes conferring resistance to sulfonamides were most abundant in the samples collected in the less industrialized area (WM-WWTP). The concentrations of *sul*1 and *sul*2 ranged from <LOD to $2.77x10^9$ gene copies/mL in wastewater (*sul* genes were detected in 86% of wastewater samples) and from $1.22x10^{10}$ to $5.23x10^{11}$ gene copies/g in sewage sludge. The

concentrations of the analyzed genes in samples from S-WWTP and WM-WWTP were highly similar. The genes encoding resistance to sulfonamides, *sul*1 and *sul*2, were characterized by the highest concentrations. The concentrations of integrase genes, *int*I1 and *int*I2, were equally high, ranging from $7.4x10^3$ to $1.7x10^8$ gene copies/mL and from $1.24x10^7$ to $3.81x10^{10}$ gene copies/g in samples from both WWTPs. Such high abundance of *sul*1 and *sul*2 genes could be related to their location on MGEs such as class 1 and class 2 integrons.

A comparison of untreated and treated wastewater samples revealed that gene concentrations were reduced by two to three orders of magnitude on average in both WWTPs. The percentage reduction in the concentrations of ARGs, integrase genes and the 16S rRNA gene reached >95% in both WWTPs.

An analysis of the concentrations of ARGs, integrase genes and the 16S rRNA gene in river water sampled upstream and downstream from the wastewater discharge point revealed that they tended to increase by one order of magnitude after treatment. A continuous inflow of ARGs from WWTPs may considerably contribute to the dissemination of antimicrobial resistance in the natural environment.

In the study described in research article 3 (Appendix No. 3 to this dissertation), Illumina deep sequencing was used to identify ARGs, MGEs and the taxonomic structure of the microbiome in wastewater and sludge samples. The databases used in the study supported the identification of 1 906 ARGs and class 1, 2 and 3 integron integrase genes. Antibiotic resistance genes from the beta-lactamase group accounted for 67.3% of all identified genes in the samples from WM-WWTP, and for 62.7% of genes from S-WWTP. A total of 1 283 genes encoding resistance to beta-lactams, assigned to 58 types, were found in the samples from WM-WWTP, and a total of 1 195 genes representing 52 types were found in the samples from S-WWTP. The study focused mostly on CRGs, i.e. $bla_{GES}$, $bla_{IMP}$, $bla_{KPC}$, $bla_{NDM}$, and $bla_{VIM}$ as well as $bla_{OXA-23}$, $bla_{OXA-24}$, $bla_{OXA-48}$ and $bla_{OXA-58}$ subtypes.

In samples from both WWTPs, the dominant beta-lactamase gene types were $bla_{OXA}$, $bla_{GES}$, $bla_{BEL}$, $bla_{CfxA}$ and $bla_{TEM}$. In the samples from WM-WWTP, these genes occurred in the following ranges: 0.064–1.415 ppm, 0.008–2.358 ppm, 0.014–0.388 ppm, 0.008–0.98 ppm and 0.008–0.758 ppm, respectively, whereas in the samples from S-WWTP, these genes occurred in the following ranges: 0.041-1.765 ppm, 0.008-1.224 ppm, 0.019-0.425 ppm, 0.01-1.458 ppm and 0.02-0.559 ppm, respectively. In samples from both WWTPs, the most abundant CRGs were the $bla_{GES}$ type and $bla_{OXA-23}$, $bla_{OXA-24}$ and $bla_{OXA-48}$ subtypes. Processes such as mechanical treatment with the use of screens and sedimentation tanks, applied in the initial stage of treatment, supported a high reduction in the abundance of carbapenemase genes from

inflowing wastewater. The *bla*$_{NDM}$ and *bla*$_{KPC}$ genes, which were present only in the influents to WM-WWTP, were eliminated from wastewater after the mechanical treatment stage.

Three MEGs, *int*I1, *int*I2 and *int*I3, were identified in the analyzed samples. In both WWTPs, *int*I1 accounted for >98% in each sample.

An analysis of taxonomic structure in the tested samples revealed the presence of 22 phyla of the domain Bacteria (82%), and three phyla of the domain Archaea (12%). Moreover, the occurrence of 17 classes representing four phyla (Proteobacteria, Firmicutes, Bacteroidetes, and Euryarchaeota) was determined, and particular emphasis was placed on three families (*Enterobacteriaceae*, *Moraxellaceae* and *Fusobacteriaceae*) whose members often produce beta-lactamases and are the causative agents of hospital infections. The dominant phyla in wastewater samples from both WWTPs were Proteobacteria, Bacteroidetes and Firmicutes. Microorganisms of the phylum Proteobacteria were most abundant in each stage of the wastewater treatment process in both WWTPs. Representatives of the above families (*Enterobacteriaceae*, *Moraxellaceae* and *Fusobacteriaceae*) were more abundant in the samples from WM-WWTP than in those from S-WWTP, whereas members of the family *Moraxellaceae* were most abundant in wastewater samples from both WWTPs. A comparison of the influents and effluents in both WWTPs demonstrated that the counts of microorganisms of the analyzed families were reduced by 85-99%.

An analysis of the prevalence of antibiotics and HMs in samples from both WWTPs (research article in Appendix No. 2 to this dissertation) revealed the highest concentrations of fluoroquinolones, including CIP (0.0128-1179.9 ng/g), NOR (<LOD-319.7 ng/g), OFX (0.0047-66.1 ng/g) and PEF (<LOD-29 ng/g), and the lowest concentrations of sulfonamides, including SDM (<LOD-1.59 ng/g), SXT (<LOD-2.98 ng/g) and ST (<LOD-1.67 ng/g).

In both WWTPs, sludge samples, compared with wastewater and river water samples, were characterized by significantly higher concentrations of most antibiotics. The obtained results confirmed that immobilization in sewage sludge is the main mechanism supporting the elimination of fluoroquinolones, tetracyclines and, to a lesser degree, SXT. Significantly lower concentrations of CIP, OFX, NOR and ST were noted in the aqueous phase after wastewater treatment. Significantly higher concentrations of SXT, TET (WM-WWTP) and ST (S-WWTP) were observed in river water sampled downstream than upstream from the wastewater discharge point.

In both WWTPs, HM concentrations were determined in the range of <LOD - 551 mg/L in liquid samples (wastewater and river water) and <LOD - 1090.8 mg/kg DM in solid samples (sludge). Liquid and solid samples from both WWTPs were most abundant in Zn whose

concentration was determined in the range of <LOD-551 mg/L and 706.4-1090.8 mg/kg DM, respectively. The samples from S-WWTP were characterized by higher HM concentrations. Attention should be paid to the differences in the concentrations of Pb in sludge samples between the analyzed WWTPs. The samples from WM-WWTP were less abundant in Pb than the samples from S-WWTP. The above could be attributed to the fact that S-WWTP is situated in an industrial region and processes more industrial wastewater. An analysis of HM concentrations in samples from both WWTPs revealed that (i) HM concentrations differed significantly between wastewater and sludge samples, (ii) the evacuation of treated wastewater did not increase HM concentrations in river water ($p>0.05$), taking into account HM concentrations in the samples collected upstream and downstream from the wastewater discharge point, (iii) the treatment-induced decrease in HM concentrations was noticeable, but not statistically significant ($p>0.05$).

The Spearman's rank correlation test demonstrated a higher number of significant correlations ($p < 0.01$) between integrase genes and genes encoding resistance to beta-lactams in the samples from WM-WWTP, which receives a higher proportion of hospital wastewater in municipal wastewater, than in the samples from S-WWTP. In the samples from WM-WWTP, significant correlations were determined between the abundance of ARGs and *int*I genes (38 for *int*I1, 33 for *int*I2 and 23 for *int*I3); in the samples from S-WWTP, the noted values were 22, 16, and 3 for *int*I1, *int*I2 and *int*I3, respectively. In the samples from WM-WWTP, the presence of strong, positive, significant correlations was observed between the abundance of the three studied integrase gene types and carbapenemase genes $bla_{GES}$, $bla_{VIM}$, $bla_{OXA-24}$, $bla_{OXA-48}$ and $bla_{OXA-58}$. The abundance of *int*I1 and *int*I2 genes was bound by weaker correlations with $bla_{GES}$, $bla_{OXA-23}$, $bla_{OXA-24}$ and $bla_{OXA-48}$ genes in the samples from S-WWTP than in those from WM-WWTP.

The number of significant positive correlations between the counts of selected microbial families (*Enterobacteriaceae, Moraxellaceae* and *Fusobacteriaceae*) and the abundance of integrase and carbapenemase genes was higher in the samples from WM-WWTP (22 correlations) than in the samples from S-WWTP (19 correlations). Significant correlations were found between the abundance of *int*I1 and *int*I2 genes and the abundance of *Enterobacteriaceae, Moraxellaceae* and *Fusobacteriaceae* in samples from both WWTPs. Significant correlations between the abundance of the *int*I3 gene and *Fusobacteriaceae* (0.44) and *Moraxellaceae* (0.51) were noted only in the samples from WM-WWTP. In samples from both WWTPs, the abundance of *Fusobacteriaceae* and *Moraxellaceae* was strongly significantly correlated with the abundance of CRGs.

*Publications:*

**Hubeny, J.**, Harnisz, M., Korzeniewska, E., Buta, M., Zieliński, W., Rolbiecki, D., Giebułtowicz, J., Nałęcz-Jawecki, G., Płaza, G., 2021. Industrialization as a source of heavy metals and antibiotics which can enhance the antibiotic resistance in wastewater, sewage sludge and river water. PLoS One 16, e0252691. https://doi.org/10.1371/journal.pone.0252691. Number of points assigned by the Ministry of Education and Science: 100, IF: 3.752.

**Hubeny, J.**, Ciesielski, S., Harnisz, M., Korzeniewska, E., Dulski, T., Jałowiecki, Ł., Płaza, G., 2021. Impact of Hospital Wastewater on the Occurrence and Diversity of Beta-Lactamase Genes During Wastewater Treatment with an Emphasis on Carbapenemase Genes: A Metagenomic Approach. Front. Environ. Sci. 9. https://doi.org/10.3389/fenvs.2021.738158. Number of points assigned by the Ministry of Education and Science: 100, IF: 5.411.

Stage III

*Aim of the study:*

The third stage of the study focused on determining the presence of antimicrobial resistance determinants in *Acinetobacter* sp. strains isolated from wastewater samples in individual stages of the treatment process and river water samples collected in three seasons (February, June, and September). A comprehensive study was carried out using classical and molecular microbiology methods (qualitative and quantitative PCR and metagenomic analysis).

*Materials and Methods:*

Samples were collected in a WWTP located in the Region of Warmia and Mazury in north-eastern Poland (WM-WWTP). In the studied WWTP, wastewater is treated by mechanical biological methods. The plant processes mainly municipal wastewater as well as wastewater from three hospitals in the city of Olsztyn. Hospital wastewater accounts for 2% of the total volume of influent wastewater. Samples of river water collected upstream and downstream from the wastewater discharge point, samples of untreated wastewater, samples of wastewater from the activated sludge bioreactor, and samples of treated wastewater were collected in late winter (February), summer (June) and fall (September).

Bacteria of the genus *Acinetobacter* were isolated from the collected samples and cultured on the CHROMagar™ Acinetobacter chromogenic medium with the addition of MDR supplement

CR102 (CHROMagar). DNA isolated from the obtained isolates (with the AX Gravity kit, A&A Biotechnology, Poland) and DNA isolated directly from wastewater and river water samples (with the FastDNA SpinKit for Feces, MP Biomedicals) were used in molecular analyses.

*Acinetobacter* strains were identified in the PCR assay. The identification of *Acinetobacter* sp. was confirmed by the detection of the *rec*A gene, and the identification of *Acinetobacter baumannii* was confirmed by the detection of the *Ab*-ITS gene. *Acinetobacter* strains were also analyzed to determine the presence of genes encoding resistance to carbapenems ($bla_{\text{IMP}}$, $bla_{\text{IMP-1}}$, $bla_{\text{VIM}}$, $bla_{\text{VIM-2}}$, $bla_{\text{NDM}}$, $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-24-like}}$, $bla_{\text{OXA-51-like}}$, $bla_{\text{OXA-58-like}}$), class 1, 2 and 3 integron-integrase genes (*int*I1, *int*I2, *int*I3) and insertion sequences (*ISAba1*) conditioning the overexpression of $bla_{\text{OXA-23-like}}$ and $bla_{\text{OXA-51-like}}$ genes in the isolates. In addition, the copy numbers of *Acinetobacter baumannii* identification genes (*Ab*-ITS) were determined in gDNA samples by qPCR with the SYBR Green fluorescent dye. The last method deployed to determine the prevalence of CRGs was "whole-genome shotgun" metagenomic sequencing conducted by Macrogen using the TruSeq DNA PCR-Free Kit.

*Results:*

A total of 258 isolates identified as *Acinetobacter* spp. were obtained in the analyses of wastewater and river water samples. Of those, 21 isolates were identified as *Acinetobacter baumannii* (8.13%). In the group of samples collected in three seasons, the samples collected in June and September were characterized by the highest number of *Acinetobacter* isolates (94 and 90, respectively), whereas 74 *Acinetobacter* isolates were obtained from February samples. The abundance of *Acinetobacter baumannii* (*Ab*-ITS gene) in environmental samples, determined by qPCR, revealed the same trend in the prevalence of the analyzed gene across seasons.

*Ab*-ITS was eliminated in 97.5-99.7% during wastewater treatment, but the quantity of *Ab*-ITS was higher in river water sampled downstream than upstream from the wastewater discharge point.

The qualitative PCR assay revealed that *Acinetobacter* spp. isolated from wastewater samples harbored nine CRGs ($bla_{\text{NDM}}$, $bla_{\text{IMP}}$, $bla_{\text{VIM}}$, $bla_{\text{OXA-23}}$, $bla_{\text{OXA-24}}$, $bla_{\text{OXA-51}}$, $bla_{\text{OXA-58}}$, $bla_{\text{IMP-1}}$, $bla_{\text{VIM-2}}$) as well as three class 1, class 2 and class 3 integron-integrase genes (*int*I1, *int*I2, *int*I3). In all isolates identified as *Acinetobacter* spp., 131 isolates (50.7%) did not harbor any ARGs, 81 isolates (31.4%) harbored one ARG, 37 isolates (14.3%) carried two ARGs, eight isolates (3.1%) harbored three ARGs, and one isolate (0.4%) carried four ARGs ($bla_{\text{IMP}}$, $bla_{\text{VIM}}$, $bla_{\text{OXA-}}$

$_{51}$ and *bla*$_{IMP-1}$). The strain harboring the highest number of ARGs was isolated from treated wastewater samples collected in September. An analysis of the isolates identified as *A. baumannii* revealed that two isolates (9.5%) did not carry any ARGs, eight isolates (38%) harbored one ARG, 10 isolates (47.6%) carried two ARGs, and one isolate (4.7%) harbored three ARGs (*bla*$_{IMP}$, *bla*$_{OXA-51}$ and *bla*$_{IMP-1}$).

In the metagenomic analysis of CRGs, the quantity of the *bla*$_{OXA-58}$ gene was determined in the range of 0.00-2.168 ppm, 0.00-2.581 ppm and 0.00-7.719 ppm in environmental samples collected in February, June and September, respectively. The *bla*$_{OXA-58}$ gene was most abundant in the studied samples, and other genes were detected sporadically.

The presence of the *ISAba1* gene related to gene overexpression was determined by qualitative PCR. A total of 41 isolates that had previously been described as *bla*$_{OXA-23}$-positive and/or *bla*$_{OXA-51}$-positive were selected for the qPCR assay. The following results were obtained: i) the presence of IS*Aba1* was confirmed in 10 out of 18 *A. baumannii* (55.6%) isolates and in 10 out of 22 *Acinetobacter* isolates (43.5%), ii) the co-existence of IS*Aba1*/*bla*$_{OXA-51}$ was observed in seven *A. baumannii* isolates (38.9%) and in five *Acinetobacter* isolates (21.7%). It should be noted that isolates harboring the IS*Aba1*/*bla*$_{OXA-51}$ complex were isolated from samples of wastewater, including treated wastewater.

*Publication:*

**Hubeny, J.**, Korzeniewska, E., Buta-Hubeny, M., Zieliński, W., Rolbiecki, D., Harnisz, M., 2022. Characterization of carbapenem resistance in environmental samples and Acinetobacter spp. Isolates from wastewater and river water in Poland. Sci. Total Environ. 822. https://doi.org/10.1016/j.scitotenv.2022.153437. Number of points assigned by the Ministry of Education and Science: 200, IF: 10.753.

Stage IV

*Aim of the study:*

The aim of the fourth stage of the study was to determine the effectiveness of the degradation of pollutants, antibiotics and ARGs in treated wastewater with the use of solar-light driven photolysis and $TiO_2$-based photocatalysis.

*Materials and Methods:*

Samples of treated wastewater were collected in a WWTP located in the Region of Warmia and Mazury, referred to as WM-WWTP in the previous stages of the study. Samples of secondary effluents were collected as 24 h composite samples. The analyzed samples (500 mL) were irradiated with the Solarbox 1500 photoreactor in triplicate. Two experimental treatments were considered: i) samples treated with solar light only, and ii) samples treated with solar light combined with a photocatalyst ($TiO_2$). An experimental sample and a control sample were used in both treatments. After treatment, the abundance of antibiotics and their TPs was determined by HPLC-MS/MS, and the abundance of ARGs, integrase gernes, genes identifying fecal contamination indicator microorganisms, and the 16S *r*RNA gene was determined by quantitative PCR.

*Results:*

In the study described in the research article in Appendix No. 5 to this dissertation, the concentrations of selected ARGs (*bla*$_{TEM}$, *bla*$_{OXA}$, *bla*$_{SHV}$ – beta-lactam resistance genes; *aac*(6')-I*b* – aminoglycoside resistance genes; *qep*A – fluoroquinolone resistance genes; *sul*1, *sul*2 – sulfonamide resistance genes; *dfr*A1 – trimethoprim resistance gene), class 1 and 2 integron-integrase genes (*int*I1, *int*I2) belonging to MGEs and genes identifying fecal contamination indicator microorganisms for the species *Enterococcus faecalis* (*efec*) and *Escherichia coli* (*uid*A) were determined by qPCR. The prevalence of *bla*$_{SHV}$, *qep*A and *dfr*A1 was not determined. Significant changes in the concentrations of the analyzed genes were noted when treated wastewater samples were compared before and after irradiation. Gene abundance was significantly reduced in wastewater samples after irradiation, both with and without the photocatalyst. The studied genes were removed in 83.8% by photodegradation, and in 89.9% with the use of the photocatalyst. The highest level of degradation was noted for *sul*1, *uid*A and *int*I1 genes.

The efficacy of photodegradation in removing antibiotics from the studied samples was also determined. A total of 16 antimicrobial compounds representing several classes were analyzed, i.e. amoxycillin, ampicillin, ciprofloxacin, enrofloxacin, levofloxacin, tylosin, clarithromycin, vancomycin, clindamycin, trimethoprim, metronidazole, sulfamethoxazole, sulfadiazine, tetracycline, doxycycline and oxytetracycline. An analysis of degradation efficiency revealed that total antibiotic removal by irradiation reached 52.4%, and it was increased to 68.5% when the photocatalyst was used. Trimethoprim and tetracycline were most susceptible to irradiation, and they were almost completely removed from the analyzed samples. These results indicate

that the applied methods supported the removal of the tested antibiotics and genes even from a complex matrix such as treated wastewater. Photocatalysis was characterized by higher removal efficiency than irradiation without $TiO_2$.

*Publication:*

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## Summary and Conclusions

The results of the presented doctoral research expand the existing knowledge about the effect of the structure of wastewater inflowing to municipal wastewater on the prevalence of ARB, ARGs MGEs and chemical contaminants such as antibiotics and HMs in wastewater, sewage sludge and river water. The high potential of photolysis and photocatalysis in removing antibiotics and antimicrobial resistance determinants from treated wastewater was confirmed. The tools used in the study included conventional microbiology methods as well as molecular microbiology techniques, bioinformatics analysis and high-performance liquid chromatography.

The results of the study supported the validation of the research hypotheses and the formulation of the following conclusions:

1. The composition of municipal wastewater treated in WWTPs significantly affects the emergence of antibiotic resistance;
2. In a highly industrialized region, the analyzed samples were characterized by higher contamination with HMs and a higher number of significant correlations between the examined micropollutants;
3. The correlations noted between integrase genes, ARGs and HMs point to the important role of integrons as MGEs in the spread of antibiotic resistance;
4. Heavy metals induce antibiotic resistance;
5. The inflow of hospital wastewater to WWTPs increased the prevalence of antimicrobial resistance determinants of particular clinical significance in wastewater samples;
6. The presence of genes encoding resistance to last-resort antibiotics associated with dangerous hospital outbreaks was noted in municipal wastewater with a higher proportion of hospital wastewater.
7. WWTPs play an important role in the dissemination of antibiotics, ARB and antimicrobial resistance determinants in the natural environment;
8. The use of additional wastewater treatment methods such as photocatalysis in the presence of $TiO_2$ may significantly increase the removal efficiency of antibiotics and antimicrobial resistance determinants from treated wastewater, and decrease the contribution of WWTPs to environmental pollution.

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